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(54) Title: COMPOUNDS AND METHODS FOR THE DIAGNOSIS, TREATMENT AND PREVENTION OF DISEASES OF CELL DEATH (57) Abstract Disclosed is the family of genes responsible for the neurodegenerative diseases, particularly Amyotrophic Lateral Sclerosis. Methods and compounds for the diagnosis, prevention, and therapy of the disease are also disclosed. Uses of the compounds in the preparation of diagnostic and therapeutic medicaments are also provided.		

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Compounds and Methods for the Diagnosis,
Treatment and Prevention
of Diseases of Cell Death

5 Statement as to Federally Sponsored Research

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10 Background of the Invention

The invention relates to cell death diseases.

Neurodegenerative diseases include familial and sporadic amyotrophic lateral sclerosis (FALS and ALS, respectively), familial and sporadic Parkinson's disease, 15 Huntington's disease, familial and sporadic Alzheimer's disease, olivopontocerebellar atrophy, multiple system atrophy, progressive supranuclear palsy, diffuse lewy body disease, corticodentatonigral degeneration, progressive familial myoclonic epilepsy, strionigral 20 degeneration, torsion dystonia, familial tremor, Gilles de la Tourette syndrome, and Hallervorden-Spatz disease. Most of the diseases are typified by onset during the middle adult years and lead to rapid degeneration of specific subsets of neurons within the neural system, 25 ultimately resulting in premature death. There is no known cure nor is there an effective therapy to slow the progression for any of the stated diseases.

Amyotrophic lateral sclerosis (ALS) is the most commonly diagnosed progressive motor neuron disease. The 30 disease is characterized by degeneration of motor neurons in the cortex, brainstem and spinal cord (Principles of Internal Medicine, 1991 McGraw-Hill, Inc., New York; Tandan et al. Ann. Neurol, 18:271-280, 419-431, 1985). Generally, the onset is between the third and sixth

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decade, typically in the sixth decade. ALS is uniformly fatal, typically within five years (Kurland et al., Proc Staff Meet Mayo Clin, 32:449-462, 1957). The cause of the disease is unknown and ALS may only be diagnosed when
5 the patient begins to experience asymmetric limb weakness and fatigue, localized fasciculation in the upper limbs and/or spasticity in the legs which typifies onset.

In ALS the neurons of the cerebral cortex and anterior horns of the spinal cord, together with their
10 homologues in some of the motor nuclei of the brain stem, are affected. The class of neurons affected is highly specific: motor neurons for ocular motility and sphincteric motor neurons of the spinal cord remain unaffected until very late in the disease. Although
15 death occasionally results shortly after the onset of the symptomatic disease, the disease generally ends with respiratory failure secondary to profound generalized and diaphragmatic weakness.

About 10% of ALS cases are inherited as an
20 autosomal dominant trait with high penetrance after the sixth decade (Mulder et al. Neurology, 36:511-517, 1986; Horton et al. Neurology, 26:460-464, 1976). In almost all instances, sporadic and autosomal dominant familial ALS (FALS) are clinically similar (Mulder et al.
25 Neurology, 36:511-517, 1986; Swerts et al., Genet. Hum, 24:247-255, 1976; Huisquinet et al., Genet. 18:109-115, 1980). It has been shown that in some but not all FALS pedigrees the disease is linked to a genetic defect on chromosome 21q (Siddique et al., New Engl. J. Med.,
30 324:1381-1384, 1991.

Parkinson's disease (paralysis agitans) is a common neurodegenerative disorder which appears in mid to late life. Familial and sporadic cases occur, although familial cases account for only 1-2 percent of the

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observed cases. The neurological changes which cause this disease are somewhat variable and not fully understood. Patients frequently have nerve cell loss with reactive gliosis and Lewy bodies in the substantia nigra and locus coeruleus of the brain stem. Similar changes are observed in the nucleus basalis of Meynert. As a class, the nigrostriatal dopaminergic neurons seem to be most affected.

The disorder generally develops asymmetrically with tremors in one hand or leg and progresses into symmetrical loss of voluntary movement. Eventually, the patient becomes incapacitated by rigidity and tremors. In the advanced stages the disease is frequently accompanied by dementia.

Diagnosis of both familial and sporadic cases of Parkinson's disease can only be made after the onset of the disease. Anticholinergic compounds, propranolol, primidone and levodopa are frequently administered to modify neural transmissions and thereby suppress the symptoms of the disease, though there is no known therapy which halts or slows the underlying progression. Deprenyl has shown some therapeutic promise.

Huntington's disease is a progressive disease which is always transmitted as an autosomal dominant trait. Individuals are asymptomatic until the middle adult years, although some patients show symptoms as early as age 15. Once symptoms appear, the disease is characterized by choreoathetotic movements and progressive dementia until death occurs 15-20 years after the onset of the symptoms.

Patients with Huntington's disease have progressive atrophy of the caudate nucleus and the structures of the basal ganglia. Atrophy of the caudate nucleus and the putamen is seen microscopically where

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there is an excessive loss of neural tissue. However, there are no morphologically distinctive cytopathological alterations which have been observed.

Although some of the characteristic mental
5 depression and motor symptoms associated with
Huntington's may be suppressed using tricyclic
antidepressants and dopamine receptor antagonists,
respectively, no therapy exists for slowing or preventing
of the underlying disease process. Huntington's disease
10 appears to map to a single locus on chromosome 4 and a
linkage test currently exists for the clinical assessment
of disease risk in presymptomatic individuals with
afflicted relatives.

Hallervorden-Spatz disease is a neurodegenerative
15 disease which affects the neurons in the region of the
basal ganglia. The symptoms generally appear during
childhood and adolescence and the disease appears with an
inheritance pattern that appears to be autosomal
recessive. Patients show abnormalities in muscle tone
20 and movement such a choreoathetosis and dystonia similar
to that seen in parkinsonism. As the disease progresses
there is increasing dementia. Death generally occurs
approximately ten years after onset.

There is no known presymptomatic diagnosis, cure
25 or treatment for Hallervorden-Spatz disease. However,
iron toxicity has recently been implicated in the
progression of this disease Greenfield, Neuropathology,
W. Blackwood & J.A.N. Corsellis, Eds. (Edinburgh; T. and
A. Constable, Ltd., 1976) pages 178-180. As a result of
30 this implication, the chelating agent deferoxamine
mesylate has been administered to patients. However,
this therapeutic approach has shown no definite benefit
(Harrison's Principles of Internal Medicine, Wilson et
al. Eds., McGraw-Hill, Inc., New York, 1991).

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Alzheimer's disease is the most important of the neurodegenerative diseases due to the high frequency of occurrence within the population and the fatal course of the disease. Two forms of the disease exist: presenile dementia, in which the symptoms emerge during middle age, and senile dementia which occurs in the elderly. Both forms of the disease appear to have the same pathology. A clear genetic predisposition has been found for presenile dementia. Familial autosomal dominant cases have been reported and the majority of individuals with trisomy 21 (Down's syndrome) develop presenile dementia after the age of 40. The familial Alzheimer's cases map to chromosomes 14, 19 and 21, with more than one locus on 21.

Olivopontocerebellar atrophy (OPCA) is a disease classification which includes a number of disorders characterized by a combination of cerebellar cortical degeneration, atrophy of the inferior olivary nuclei and degeneration and disappearance of the pontine nuclei in the basis pontis and middle cerebellar peduncles. Autosomal dominant inheritance is characteristic in most families. In one family, termed the Schut family, genetic linkage has been shown to chromosome 6. An excess of glutamate has been implicated as the causative agent in this disease. A gene with an expanded CAG trinucleotide repeat [causes one form of OCPA] has now been identified and eluted sequencing can be used for diagnosis (Orr et al., *Nature Genetics* 4:221-226, 1993).

The human superoxide dismutases are actually at least three different enzymes: cytosolic Cu/Zn superoxide dismutase encoded by the SOD1 gene on chromosome 21 (Levanon et al., *EMBO J.* 77-84, 1985 and Hjalmarsson et al., *P.N.A.S.* 84:6340-6344, 1987); mitochondrial superoxide dismutase encoded by the SOD2 gene on

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chromosome 6 (Wispe et al., Biochim. Biophys. Acta. 994:30-36, 1989); and extracellular superoxide dismutase encoded by the SOD3 gene on chromosome 4 (Hjalmarsson, supra). SOD1, for example, is a homodimeric

5 metalloenzyme that catalyzes the dismutation of the toxic superoxide anion O_2^- to O_2 and H_2O_2 . The major function of the superoxide dismutase is to eliminate O_2^- resulting from aerobic respiration. As a class of polypeptides present in most living organisms, these enzymes are
10 differentially associated with different metals including iron, manganese, copper and copper-zinc.

In Guam an inherited disease termed Parkinsonism-dementia complex has been described. Clinical, pathological and familial studies have indicated that
15 this disease is a clinical variant of the local form of ALS. Cases of presenile dementia in the absence of ALS or Parkinsonism have also been observed in this population (Kurland et al. In Norris FH Jr. and Kurland LT eds. Motor Neuron Diseases: Research on amyotrophic
20 lateral sclerosis and related disorders. NY: Grune & Stratton, 1969; 84:28-50; Hirano et al., Brain 84:642-661, 1961; and Hirano et al., Brain 84:662-679, 1961).

Hallewell et al. (US Patent No. 5,066,591) describe methods and compositions for the production of
25 human copper/zinc superoxide dismutase polypeptides in microorganisms.

Hallewell (US Patent No. 5,084,390) describe recombinant Cu/Zn superoxide dismutase polymers having an extended in vivo half-life composed of SOD monomers
30 covalently coupled to each other.

Bruice (International Patent Application No. PCT/US91/06558) describe synthetic enzymes that mimic catalytic activity of superoxide dismutase.

Bracco et al. (P.S.E.B.M. 196:36-41, 1991) have
35 measured the levels of superoxide dismutase in the

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cerebral spinal fluid of patients with age-related neurodegenerative disorders including ALS, Alzheimer's disease, and a reference group of normal subjects.

Bracco et al. report that the superoxide dismutase

5 activity was found to increase with the age of the subject while no significant correlation was found in the ALS and Alzheimer's disease patients. The activity mean values were found to be significantly lower in patients with ALS and Alzheimer's disease.

10 Liu et al. (Amer. Physiol. Soc. H589-H593, 1989) describe the administration of polyethylene glycol-conjugated superoxide dismutase and catalase to reduce ischemic brain injury in rats.

 Olanow (Ann Neurol. 32:52-59, 1992) have proposed
15 free radicals as the cause of neuronal injury in several neurological disorders, including Parkinson's disease and ischemic brain injury.

Summary of the Invention

 We have discovered that mutations in superoxide
20 dismutase cause familial amyotrophic lateral sclerosis. Accordingly we have determined methods for the diagnosis and treatment of amyotrophic lateral sclerosis and other cell death disease, particularly neurodegenerative diseases. Methods are provided for treating familial
25 amyotrophic lateral sclerosis and amyotrophic lateral sclerosis as well as other cell death diseases which are the result of decreased SOD activity, altered SOD enzymatic activity, and altered SOD physical characteristics. In addition, therapeutics for diseases
30 caused by alterations in the SOD biochemical pathway are provided.

 In the first aspect, the invention features methods of diagnosing an increased likelihood of developing cell death disease in a patient. The methods
35 include analyzing the DNA of the patient to determine

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whether the DNA contains a mutation in SOD coding sequence, such a mutation being an indication that the patient has an increased likelihood of developing a cell death disease. The methods may be used to diagnose a
5 cell death disease, particularly neurodegenerative disease, more particularly Parkinson's disease, Huntington's disease, Alzheimer's disease, Hallervorden-Spatz disease, olivopontocerebellar atrophy, multiple system atrophy, progressive supranuclear palsy, diffuse
10 lewy body disease, corticodentatonigral degeneration, progressive familial myoclonic epilepsy, strionigral degeneration, torsion dystonia, familial tremor, Gilles de la Tourette syndrome, and Hallervorden-Spatz disease, and ALS which is familial, sporadic typical, or atypical
15 in nature. These methods may also be used for the diagnosis of a SOD related disease in a fetus.

The methods may include amplifying a SOD-encoding gene of the patient using SOD-specific primers, and then analyzing the amplified gene. The DNA may be analyzed by
20 nucleotide sequencing, SSCP analysis, RFMP, heteroduplex analysis or RFLP analysis. The amplifying may be carried out by PCR reaction, by reverse transcriptase PCR or by any other method available to obtain a sufficient amount of DNA.

25 The primer sequence may be derived from SOD1 nucleic acids, SOD2 nucleic acids, SOD3 nucleic acids or nucleic acids from any other human SOD gene.

Antibodies which recognize familial amyotrophic lateral sclerosis SOD polypeptides but fail to recognize
30 wild-type SOD may also be used for the diagnosis of familial amyotrophic lateral sclerosis in patients.

In the second aspect, the invention features kits for the diagnosis of a cell death disease in a patient. The kits may include one or more SOD gene-specific PCR
35 primers or antibodies recognizing the SOD polypeptides.

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The PCR primers may include an SOD1-specific nucleic acid sequences, SOD2-specific nucleic acid sequences, SOD3-specific nucleic acid sequences. These kits may be used to diagnose any of the above-referenced diseases.

5 Kits which include antibodies which specifically recognize mutant SOD polypeptides present in amyotrophic lateral sclerosis patients are part of the kits of the inventor.

 In the third aspect, the invention features
10 methods of treating a patient with a disease involving a mutant SOD encoding gene or environmentally induced ALS. The methods include administering to the patient an antioxidant, effective to reduce the symptoms the disease in the patient. The antioxidant may be vitamin C,
15 vitamin E, a lazaroid, BHA, BHT, Beta-carotene, urate, bilirubin, glutathione, dimercaptol lutein, upiguinol-10, dithiothreitol, mercaptan, a sulfa compound, methionine, cystein, or N-acetyl cysteine, or any other antioxidant which reduces the level of toxic compounds in
20 the affected cells. Most preferably, the antioxidant is vitamin C or vitamin E.

 Also included are methods of treating a patient with a disease involving a mutant SOD encoding gene or a patient with sporadic ALS due to environmental causes
25 which include administering to the patient SOD polypeptide, in an amount effective to reduce the symptoms of said disease in said patient. The SOD polypeptide may be SOD Cu/ZnSOD, mSOD, ecSOD, or derivatives, as described below.

30 Methods of treating the above patients may also include administering to the patient a chelating agent, e.g. desferoxamine, or transgene including a nucleotide sequence encoding a SOD polypeptide e.g., a nucleotide sequence which encodes the Cu/ZnSOD polypeptide, the mSOD
35 polypeptide, or ecSOD polypeptide. Preferably the

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nucleotide sequence encodes Cu/ZnSOD or mSOD, and most preferably the nucleotide sequence encodes Cu/ZnSOD.

Also included in the invention is a method for treating a patient with a disease involving a mutant SOD
5 encoding gene. This method includes first identifying a mutant SOD polypeptide-encoding gene in the DNA of the patient, and, second, administering to the patient a therapeutic amount of the anti-sense RNA homolog of a gene encoding a SOD polypeptide. The polypeptide may be
10 wild-type SOD or a polypeptide encoded by the mutant SOD-encoding gene.

Also included is a method for treating a patient with a disease involving a mutant SOD encoding gene, wherein the mutant SOD polypeptide-encoding gene in the
15 DNA is identified in the patient, and a therapeutic amount of a transgene encoding the wild-type homolog of the mutant SOD polypeptide is administered.

Further included is a method for treating a patient with a disease involving a mutant SOD-encoding
20 gene, which comprises identifying the mutant SOD polypeptide-encoding gene in the DNA of the patient, and administering to the patient a therapeutic amount of a transgene encoding the anti-sense homolog of said wild-type SOD RNA.

25 Also a part of the invention is a method of treating a patient with a disease involving a mutant SOD encoding gene by administering to the patient an antibody which is sufficient to partially inactivate said mutant SOD polypeptide.

30 A method of treating a patient with a disease involving a mutant SOD encoding gene or a patient with sporadic ALS due to environmental causes either of whose disease is caused at least in part by excess SOD activity by administering to the patient an inhibitor of wild-type

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SOD, such as those provided herein, is a part of the invention.

A method of treating a patient with a disease involving a deleterious mutant SOD encoding gene or a patient with sporadic ALS due to environmental causes by the administering of a mutant SOD polypeptide with increased SOD enzymatic activity compared to wild-type SOD is also included as a part of the invention. Such a patient may also be treated by administering a nucleotide sequence encoding a non-wild-type therapeutic SOD polypeptide mutant different from and capable of inhibiting the deleterious SOD polypeptide. As in all methods, this SOD polypeptide may be a fragment of SOD, an analog of SOD, or a non-peptide mimetic of SOD.

Further included is a method of treating a patient with a disease involving a mutant SOD encoding gene by administering to the patient a compound which participates in a biochemical pathway involving a SOD polypeptide. These compounds may include glutathione peroxidase, catalase, or nitric oxide synthase. Specifically, peroxide-reducing polypeptides may be administered, as described below.

The invention also includes methods of treating or preventing ALS and FALS by the administration of inhibitors and agonists of SOD. This method is appropriate in patients in whom SOD gene which encodes a polypeptide which confers altered or increased SOD enzymatic activity. The chelating agent may be disferoxamine, EDTA, EGTA, DETC, BCDA, penicillamine, tetracycline, a metallothionein protein or an apo-metal binding protein. The metallothionein protein may be a yeast copper metallothionein. The apo-metal binding protein may be one or more of apo-superoxide dismutase, hemoglobin, myoglobin, or plastocyanin.

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Any of the methods described herein may be used alone or in combination. For example, it may be desirable to administer one or more antioxidants in combination with a chelating agent and or a SOD polypeptide therapeutic.

Any of the following diseases may be treated using one or more of the above methods: a cell death disease, particularly a neurodegenerative disease, more particularly Parkinson's disease, Huntington's disease, Alzheimer's disease, Hallervorden-Spatz disease, olivopontocerebellar atrophy, multiple system atrophy, progressive supranuclear palsy, diffuse lewy body disease, corticodentatonigral degeneration, progressive familial myoclonic epilepsy, strionigral degeneration, torsion dystonia, familial tremor, Gilles de la Tourette syndrome, and ALS which is familial, sporadic typical, or atypical in nature.

In a fourth aspect, the invention features antibodies reactive with a FALS polypeptide but not significantly reactive with a wild-type SOD polypeptide. These antibodies may be monoclonal or polyclonal and may be obtained by subtractive techniques. The antibodies may be sufficient for the inactivation of a SOD polypeptide.

These antibodies may be used as stated above to diagnose ALS in a patient by contacting a blood sample of said patient with the antibody.

In a fifth aspect, the invention features methods of treating a patient with a neoplasm by administering to the patient a FALS polypeptide. A patient with a neoplasm, may also be treated by the administration of transgene encoding an FALS polypeptide.

In the sixth aspect, the invention features a transgenic non-human animal whose somatic and germ cells contain a transgene for a disease-causing mutant SOD

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polypeptide having a nucleic acid sequence encoding a disease causing SOD polypeptide in an expressible genetic construction. The animal may be a mouse, a worm, or any other animal useful for research or drug development.

5 In the seventh aspect, the invention features a bacterial or yeast cell containing purified nucleic acid derived from a FALS gene.

 The eighth aspect, the invention features purified DNA encoding a purified FALS polypeptide, purified RNA
10 encoding a purified FALS polypeptide, and purified FALS polypeptide.

 A ninth aspect of the invention is the use of any of the methods or compounds of the invention which do not solely depend upon the physical properties of a mutant
15 SOD polypeptide for the treatment of a disease of cell death which is the result of a mutation or imbalance in a component of the SOD pathway other than the SOD polypeptide. For example, treatment of diseases due to defects in the production or function of glutathione
20 peroxidase, catalase and nitric oxide synthase. Methods useful for the treatment of these disorders include administration of wild-type and mutant SOD, anti-sense RNA to SOD encoding sequences, use of antibodies to wild-type SOD, and use of analogs and inhibitors of compounds
25 in the SOD pathway.

 More specifically, the invention provides therapies using Cu/Zn superoxide dismutase (Cu/ZnSOD), mitochondrial superoxide dismutase (mSOD), or extracellular superoxide dismutase (ecSOD) (Fig. 2-4 and
30 SEQ ID NOS: 1-3, respectively), as well as other naturally occurring superoxide dismutase polypeptides. Also included are: allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes under high (e.g., washing at 2xSSC at 40 C with a probe
35 length of at least 40 nucleotides) stringency conditions

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to naturally occurring Cu/Zn SOD, mSOD, or ecSOD-encoding nucleotide sequences, i.e. SOD1, SOD2, or SOD3; for other definitions of high and low stringency see Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989, 6.3.1 - 6.3.6, hereby incorporated by reference). The term "SOD polypeptide" also includes chimeric polypeptides that include Cu/ZnSOD, mSOD, or ecSOD together with unrelated sequences.

The invention also includes any biologically active fragment or analog of Cu/ZnSOD, mSOD or ecSOD. By "biologically active" is meant possessing therapeutically useful superoxide reducing activity which is characteristic of the Cu/ZnSOD, mSOD, or ecSOD polypeptides shown in Figs. 2-4 (SEQ ID NOS: 1-3).

15 Therapeutically useful activity of a Cu/ZnSOD, mSOD or ecSOD fragment or Cu/ZnSOD, mSOD, or ecSOD analog, can be determined in any of a variety of Cu/ZnSOD, mSOD or ecSOD assays. For example, those assays described in Wayne and Fridovich (Analytical Biochemistry, 161: 559-566 (1987)),

20 McCord and Fridovich (J. of Biol. Chem., 244: 6049-6055 (1969)), and Salin and McCord (J. of Clin. Invest., 54:1005-1009 (1974)) may be used to determine superoxide dismutase activities of Cu/ZnSOD, mSOD or ecSOD. A Cu/ZnSOD, mSOD or ecSOD analog possessing, most

25 preferably 90%, preferably 40%, or at least 10% of the activity of a wild-type or mutant Cu/Zn SOD, mSOD, or ecSOD polypeptide (shown in Figs. 2-4; SEQ ID NOS: 1-3), in any in vivo or in vitro Cu/ZnSOD, mSOD or ecSOD assay (e.g., those described herein) is considered biologically

30 active and useful in the methods of the invention.

Preferred analogs include 155-amino acid Cu/Zn SOD, 222 amino acid mSOD, or 240 amino acid ecSOD (or biologically active fragments thereof) whose sequences differ from the wild-type sequence only by conservative

35 amino acid substitutions, for example, substitution of

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one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not destroy the

5 polypeptide's relevant biological activity as measured using in vivo or in vitro (e.g., those described above). Preferred analogs also include Cu/ZnSOD, mSOD, or ecSOD (or biologically active fragments thereof) which are modified for the purpose of increasing peptide stability;

10 such analogs may contain, for example, one or more desaturated peptide bonds or D-amino acids in the peptide sequence.

Analogues can differ from naturally occurring Cu/ZnSOD, mSOD, or ecSOD polypeptides by amino acid

15 sequence differences or by modifications that do not involve sequence, or by both. Analogs useful for the methods of the invention will generally exhibit at least 65%, more preferably 80%, even more preferably 90%, and most preferably 95% or even 99%, homology with all or

20 part of a naturally occurring Cu/ZnSOD, mSOD, or ecSOD sequence. The length of comparison sequences will generally be at least about 15 amino acid residues, preferably more than 40 amino acid residues. Modifications include *in vivo*, or *in vitro* chemical

25 derivatization of polypeptides, e.g., acetylation, glycosylation, or carboxylation. Also embraced are versions of the same primary amino acid sequence that have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

30 Analogs can differ from naturally occurring Cu/ZnSOD, mSOD, or ecSOD polypeptides by alterations of their primary sequence. These include genetic variants, both natural and induced. Also included are analogs that include residues other than naturally occurring L-amino

35 acids, e.g., D-amino acids or non-naturally occurring or

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synthetic amino acids, e.g., β or γ amino acids. Alternatively, increased stability may be conferred by cyclizing the peptide molecule.

The invention also provides methods of using SOD polypeptides (or nucleotide sequences encoding polypeptides) which are obtained from other living organisms which are found to synthesize superoxide dismutases, e.g., E.coli, Saccharomyces cerevisiae, and C. elegans. Useful mutants of such SOD polypeptides are those which have increased stability or other desirable properties.

The invention also includes therapeutic uses of polypeptides (or nucleotide sequences encoding polypeptides) which are substantially (at least 70%) homologous to wild-type SOD polypeptides or genes. "Homologous" refers to the sequence similarity between two polypeptides or nucleic acids. When a position in both of the two compared sequences is occupied by the same base or amino acid monomeric subunit, e.g., if a position in each of the two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences. For example, if 6 of 10 of the positions in two sequences are homologous, then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC are 50% homologous.

The invention also includes uses of the compounds described herein in the manufacture of medicaments to be used in the diagnosis and treatment of patients with any of the cell death diseases.

Substantially pure Cu/ZnSOD, mSOD, and ecSOD polypeptides can be produced in quantity using standard recombinant DNA-based techniques. Thus, recombinant

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Cu/ZnSOD, mSOD2, or ecSOD polypeptides can be administered with a pharmaceutically acceptable diluent, carrier, or excipient, in unit dosage form.

Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer the polypeptide to patients suffering from or presymptomatic for a disease of cell death.

Further included as an aspect of the invention are the FALS-SOD polypeptides, e.g., those polypeptides encoded by the nucleic acid of patients with FALS due to a SOD mutation. Also included are the nucleic acids which encode these mutant polypeptides. Also included as an aspect of the invention are antibodies, particularly monoclonal antibodies, which are reactive with FALS-SOD polypeptides.

In addition to substantially full-length polypeptides, the invention also includes biologically active fragments of the polypeptides. As used herein, the term "fragment", as applied to a polypeptide, will ordinarily be at least about 10 contiguous amino acids, typically at least about 20 contiguous amino acids, more typically at least about 30 contiguous amino acids, usually at least about 40 contiguous amino acids, preferably at least about 50 contiguous amino acids, and most preferably at least about 60 to 80 or more contiguous amino acids in length. Fragments of Cu/ZnSOD, mSOD, or ecSOD can be generated by methods known to those skilled in the art. The ability of a candidate fragment to exhibit a biological activity of Cu/ZnSOD, mSOD, or ecSOD can be assessed by methods described below. Also included are Cu/ZnSOD, mSOD, or ecSOD polypeptides containing amino acids that are normally removed during protein processing (for example, the leader sequence of ecSOD), including additional amino acids that are not required for the biological activity of the polypeptide,

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or including additional amino acids (if any) that result from alternative mRNA splicing or alternative protein processing events.

The invention also provides methods of using SOD polypeptides (or nucleotide sequences encoding polypeptides) which are obtained from other living organisms which are found to synthesize superoxide dismutases, e.g., E.coli, Saccharomyces cerevisiae, and C. elegans. Useful mutants of such SOD polypeptides are those which have increased stability or other desirable properties.

The invention also includes therapeutic uses of polypeptides (or nucleotide sequences encoding polypeptides) which are substantially (at least 70%) homologous to wild-type SOD polypeptides or genes. "Homologous" refers to the sequence similarity between two polypeptides or nucleic acids. When a position in both of the two compared sequences is occupied by the same base or amino acid monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The homology between two sequences is a function of the number of matching or homologous positions share by the two sequences. For example, if 6 of 10 of the positions in two sequences are homologous, then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGCC are 50% homologous.

Substantially pure Cu/ZnSOD, mSOD, and ecSOD polypeptides can be produced in quantity using standard recombinant DNA-based techniques. Thus, recombinant Cu/ZnSOD, mSOD2, or ecSOD polypeptides can be administered with a pharmaceutically acceptable diluent, carrier, or excipient, in unit dosage form.

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Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer the polypeptide to patients suffering from or presymptomatic for a disease of cell death. A

5 "substantially pure" preparation of a polypeptide is a preparation which is substantially free (e.g., to the extent required for formulating Cu/ZnSOD, mSOD2, or ecSOD into a therapeutic composition) of the proteins with which it naturally occurs in a cell.

10 The formulations of the invention can be administered for example, by parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or
15 oral administration.

Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders,
20 nasal drops, or aerosols.

Methods well known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences". Formulations for parenteral administration may, for example, contain
25 excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used
30 to control the release of present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain
35 excipients, for example, lactose, or may be aqueous

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solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally.

5 Toxic SOD mutants and nucleotide sequences encoding such polypeptides can be formulated by any of the above methods for use as therapies for diseases of cell proliferation, e.g., cancer.

Other features and advantages of the invention
10 will be apparent from the following detailed description, and from the claims.

Detailed Description

The drawings will first be briefly described.

Drawings

15 Figs. 1A through 1C are a set of diagrams illustrating the single-strand conformational polymorphism and sequence analysis of SOD1 exons from FALS-affected individuals.

Fig. 1A is an autoradiogram of single strand
20 conformational polymorphism banding patterns for SOD-1 exons 2(top) and 4(bottom). "N" designates DNA from normal individuals.

Fig. 1B is a sequence analysis of SOD1 exons 2 and
4 in genomic FALS DNA.

25 Fig. 1C is a comparison of amino acid sequences from exons 2 and 4 of normal Cu/Zn SOD1 obtained from diverse species, as noted.

Fig. 2 is the genomic sequence of SOD1 and
Cu/ZnSOD polypeptide (SEQ ID No. 1).

30 Fig. 3 is the cDNA sequence of SOD2 and mSOD polypeptide (SEQ ID No. 2).

Fig. 4 is the cDNA sequence of SOD3 and the ecSOD
polypeptide (SEQ ID No. 3).

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Fig. 5 is a list of primers useful for the diagnosis of diseases linked to the SOD1 nucleic acid sequences.

Fig. 6 is a list of primers for reverse transcriptase PCR for the detection and diagnosis of SOD2 and SOD3 linked diseases.

Fig. 7 is a diagram of the pathways for the SOD superoxide dismutase enzymes.

Figs. 8A through 8C. Fig. 8A is single-strand conformational polymorphisms for exon 1 SOD1 DNA amplified from a normal individual (lane N) and a patient with FALS carrying an exon 1 mutation (lane 127).

Fig. 8B is sequence analysis from amplified DNA from patient in family 127 with exon 1 mutation. The mutation changes a C to a T in one allele; thus, the patient is heterozygous and has both a C and a T in this double-strand sequence at that base pair (arrow).

Fig. 8C is multi-species comparison of SOD1 protein sequences corresponding to exon 1. The exon 1 mutation substitutes a valine for an alanine that is completely conserved in all of the indicated species. Sources of sequences were human (Levanon et al. (1985) *EMBO J.*, 4, 77-84), bovine (Hallewell et al. (1991) *Biochem. Biophys. Res. Commun.* 181, 474-480), mouse (Benedetto et al. (1991) *Gene* 99, 191-195), *Xenopus* (Montesano et al. (1989) *Eur. J. Biochem.* 186, 421-426), *Drosophila* (Seto et al. (1989) *Gene* 75, 85-92), tomato (Perl-Treves et al. (1988) *Plant Mol. Biol.* 11, 609-623), maize (Cannon et al. (1987) *Isozymes Curr. Top. Biol. Med. Res.* 14, 73-81), *Neurospora* (Chary et al. (1990) *J. Biol. Chem.* 265, 18961-18967), and others (Hjalmarsson et al. (1987) *Proc. Natl. Acad. Sci. USA* 84, 6340-6344).

Figs. 9A and 9B are *In situ* hybridization of 51-mer from coding sequence of human SOD1 to anterior gray matter of normal human lumbosacral spinal cord. Both in

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Fig. 9A dark and Fig. 9B bright field, there is strong hybridization of probe to large motor neurons.

Fig. 10 is immunostaining of normal human lumbosacral spinal cord with monoclonal antibodies to SOD1 (Panels A and C) and neurofilament heavy chain (Panels B and D). At low power, the anterior horn shows numerous, heavily staining SOD1-positive cells which at high power have the dimensions and morphology of anterior horn cells. Another section reveals prominent neurofilament staining in this population of neurons, again with the morphology of motor neurons.

Fig. 11 is immunostaining of lumbosacral spinal cord from a patient with an exon 1 mutation in SOD1 using monoclonal antibodies to SOD1 (Panels A and D) and neurofilament (Panels B and E) as well as cresyl violet staining (Panels C and F). The tissue illustrated is from anterior horn (Panels A, B and C) and intermediolateral gray matter including Clarke's column (Panels D, E and F). In this cord, motor neurons were severely involved at death, as indicated by reduced numbers of this cell type (Panels B and C) compared to normal spinal cord. Little SOD1 staining is evident in the anterior gray matter; there may be one residual, pyknotic motor neuron with some anti-SOD1 reactivity (panel A, upper left). In contrast, numerous neurons in Clarke's column are evident by immunostaining with SOD1 (Panel D) and neurofilament (Panel E) as well as by cresyl violet staining (Panel F).

Fig. 12 is an illustration of the single-strand conformation polymorphism and sequence analysis of exon 4.

Fig. 13 is an illustration of polymorphism in intron 2 of SOD.

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I. Identification of a Class of Genetic Defects
Causing Cell Death.

Our work has shown that the SOD1 gene is specifically altered in individuals with familial ALS; based on this work, we believe that physical or enzymatic alterations in the mutant SOD polypeptides plays a fundamental role in the etiology of diseases of cell death, particularly neurodegenerative diseases such as ALS.

10 It is a striking fact that a majority of neurodegenerative diseases, ranging from Alzheimer's disease to Parkinson's disease, have a similar profile for onset and progression. These observations support the thesis that a similar mechanism forms the underlying
15 basis of all of these diseases. Our discovery that abnormal SOD is a causative agent in diseases of cell death provides a heretofore missing disease mechanism.

II. Pathogenesis of abnormal SOD1

Figure 1C is a comparison of amino acid sequences
20 encoded by exons 2 and 4 of Cu/ZnSOD of several disparate organisms: (human [Levanon et al., EMBO, J. 77-84, 1985; Hjalmarsson et al., Proc. Natl. Acad. Sci., 84:6340-6344, 1987]; mouse [Bewley, Nucl. Acids Res. 16:2728, 1988]; Onchocerca volvulus [Henkle et al., Infect. Immun.
25 59:2063-2069, 1991]; tomato [Perl-Treves et al., Plant Molec. Biol. 11:609-623, 1988]; Saccharomyces cerevisiae [Bermingham-McDonogh et al.; Proc. Natl. Acad. Sci. USA 85:4789-4793, 1988], all others [Hjalmarsson et al., Proc. Natl. Acad. Sci. 84:6340-6344, 1987]). The
30 aberrant FALS residues we discovered are denoted at the top of Fig. 1C; their corresponding positions in the coding sequence are indicated at the bottom of the Figure.

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We believe that in addition to causing a decrease in the level of cellular protection against oxygen free radicals, the FALS-associated mutations in SOD1 may confer novel properties on the corresponding mutant polypeptides. These novel properties would account for the dominant nature of the disease. Specifically, we believe that mutations in FALS SOD polypeptides confer novel chemical properties to the polypeptides so as to make them directly toxic, e.g., by destabilizing the folding of the polypeptide and causing it to adopt unstable (e.g., susceptible to proteolysis) or insoluble conformations. The mutant polypeptides may, for example, cause disease by impeding the peptide degradation systems of the cell, creating phenotypes, for example peptides which have a toxic biological function failing to efficiently sequester the copper or zinc which are toxic in elevated concentrations actively sequestering metals such as aluminum or cadmium, or by any other mechanism whereby the novel physical or chemical properties of the ALS-SOD polypeptide ultimately kills the cell. It is possible that two or more of these processes may act in a concerted fashion; they are not mutually exclusive. An example of a disease and a gene with mutations that confer such novel chemical properties on the corresponding mutant polypeptide is retinitis pigmentosa and rhodopsin (Sung et al., *PNAS U.S.A.* 88:6481-6485, 1991).

Additionally, the FALS-associated mutations in SOD1 may confer novel enzymatic properties to the corresponding mutant polypeptides, such as catalyzing the synthesis of novel compounds or the synthesis of unusual quantities of compounds which are normally benign. Either phenomenon may occur by interaction of mutant SOD with atypical substrates, such as nitric oxide. The newly created compounds could be directly toxic, such as

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free radical compounds or agonists/competitors of other enzymes, could be reactive intermediates that lead to formation of toxic compounds, or could be toxic due to the synthesis of an elevated quantity. This enzymatic toxicity may act alone or in concert with chemical toxicity to kill the cell.

Furthermore, the mutations in ALS-SOD polypeptides are likely to interfere with the normal SOD function of eliminating certain oxygen free radicals (e.g., superoxide) from the cellular environment. A survey of a sample derived from individuals with a variety of different mutations in SOD indicates that there is a loss of in most cases 10%, in some cases 25%, and only rarely more than 50% superoxide free radical elimination from the cell. This loss in free radical scavenging activity may contribute, wholly or in part, to the observed cellular toxicity in ALS. The reduction in oxygen free radical scavenging ability of the mutant SOD polypeptides can act in concert with the chemical and/or enzymatic toxicity of the mutant SOD polypeptides to kill the cell. In any potential treatment for the mitigation or cure of ALS, it is desirable to restore the oxygen free radical scavenging ability of the cell, either by supplementation with functional SOD polypeptide to normal or supranormal levels, or by use of compounds (such as antioxidants) capable of elimination of free radicals. The therapeutic restoration of free radical scavenging in the cell is desirable alone or in combination with other therapies for treatment of ALS.

Another possibility is that the mutations in SOD1 decrease or eliminate SOD1 activity. However, most loss-of-function mutations cause a recessive rather than a dominant effect (Muller In Proceedings of the Sixth International Congress of Genetics, pp 213-255, 1932; Park et al., Genetics 113:821-852, 1986). Exceptions can

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arise in proliferating cells that allow a somatic mutation to cause a loss of function of the second allele of the gene, as in the case of retinoblastoma (Dryja et al., Nature 339:556-558, 1989).

5 An additional hypothesis is that the putative FALS mutations have a dominant-negative effect (Herskowitz, Nature 219-222, 1987) such that the mutant SOD1 protein not only is functionally defective but also inhibits the function the normal SOD1 protein expressed from the
10 normal allele. Consistent with the increased activity hypothesis, both Ile-113 and Leu-106 residues are thought to be involved in forming hydrogen bonds important for the increased thermostability of a mutant form of SOD1 (Parge et al., Proc. Natl. Acad. Sci. USA 89:6109-6113,
15 1992); it is plausible that SOD1 proteins with amino acid changes at these residues are of increased stability and hence of increased activity. Consistent with the dominant-negative hypothesis, one of the sites abnormal in FALS patients, Ile-113, has been implicated in
20 hydrogen bond formation between SOD1 monomers (Kitagawa et al., J. Biochem 109:477-485, 1991); the normal and mutant proteins may combine to form an inactive heterodimer.

III. Diagnostics for Neurodegenerative disorders.

25 Neurodegenerative disorders may be diagnosed in a patient using the primers provided in Figure 5 for the SOD1 gene and in Figure 6 for the SOD2 and SOD3 genes. These primers, or other primers derived from the SOD genes, may be used to identify SOD mutations. For
30 example, diagnosis of individuals with neurodegenerative diseases resulting from mutations in the SOD1 gene may be performed using the techniques provided in the examples, below. Mutations in SOD2 and SOD3 may be diagnosed using the primers which are provided (or any primers which are

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derived from the SOD2 or SOD3 genes) in combination with the technique of reverse transcriptase PCR (Kawasaki and Wang, PCR Technology Ehrlich, Ed. (New York, Stockton Press, 1989) pages 89-98. Following amplification of the target DNA, SSCP and/or sequence analysis may be performed. It is desirable to compare the sequenced mutation to the equivalent sequences from affected and unaffected relatives in the case of familial diseases. In cases which do not appear to be familial, the mutation is compared to mutations previously observed in the affected population. Correlation with affected relatives, the diseased population, and residues which are conserved through evolution provide an additional measure of certainty useful for a definitive diagnosis.

Neurodegenerative diseases may also be diagnosed using restriction fragment length polymorphisms or any other diagnostic technique involving the detection of nucleotide changes in the SOD genes, e.g., RFMP, and heteroduplex analysis. Knowing the sequences of the SOD genes, one skilled in the art may design combinations of DNA probes and restriction enzymes to determine the afflicted individuals in a FALS (or other inherited SOD disease) family. See also the example, below.

IV. Therapies for Diseases involving a mutant SOD encoding gene.

On the basis of our findings, we conclude that toxicity caused by oxygen free radicals is a primary pathogenetic mechanism for motor neuron death in FALS and sporadic ALS. Therapeutic measures that diminish this toxicity will blunt the devastating course of these diseases. These therapeutic approaches are also appropriate for the treatment of presymptomatic individuals with defined SOD mutations as well as symptomatic individuals.

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A dominant inheritance pattern is seen in all FALS pedigrees. A dominant phenotype may be conferred by the gain of a novel function by the mutant SOD polypeptide. This gain of function may include alterations of the

5 physical properties, chemical properties, or the enzymatic properties of the mutant SOD polypeptide. For example, dominant negative effects in which the mutant SOD polypeptides inactivate wild type SOD polypeptides by causing loss of activity or stability of a multimeric

10 complex (such as the SOD homodimer) are possible mechanisms of pathogenesis. It is also possible that mutant SOD polypeptides decrease or, in rare instances, increase the activity of the enzyme. These alterations in activity level may occur when the mutant SOD is either as

15 part of a heteromeric complex including mutant and wild-type SOD subunits or as homomeric mutant subunit complex.

i) Administration of SOD polypeptides.

Wild-type SOD polypeptides may be administered to patients with a dominant negative SOD mutation which

20 lowers effective SOD levels in the affected tissue.

Mutant ALS SOD polypeptides identified in FALS patients or created in the laboratory and different from those present in the affected individual may be administered to patients with either dominant negative or

25 gain of function type SOD mutations. Useful polypeptides for this method of complementation are those which, when added to the SOD polypeptide isolated from the affected patient, negates the alteration in SOD physical or enzymatic activities conferred by the mutant SOD

30 polypeptide.

Administration of mutants with increased SOD activity may be used as a method of treating individuals with lowered SOD activity. Such mutants may be naturally occurring, e.g., FALS polypeptides, or constructed in the

35 laboratory. For example, those mutants described by

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Parge et al. (P.N.A.S. 89:6109-6113 (1992)) or Getzoff et al. (Nature 358:347-351 (1992)) may be used. FALS polypeptides or nucleic acids altered in the laboratory for therapeutic use may also be administered.

5 Proteins in which the SOD polypeptide is fused to a ligand may be used for the purpose of stabilizing and/or targeting the useful SOD polypeptides. A fusion protein consisting of a SOD polypeptide, fused to, for example, tetanus toxin, calcium channel blocking agents,
10 transferrin, poliovirus epitopes, neuropeptide fragments, or steroid hormone androgens, or a fragments thereof which are sufficient to target the SOD polypeptide to the motor neurons of an ALS patient may be used.

Proteins which are part of the SOD biochemical
15 pathway may be administered as therapeutics for diseases of cell death, particularly ALS and FALS.

ii) Administration of antioxidants

In the SOD biochemical pathway, a decrease in SOD activity results in an increase in the concentration of
20 $O_2\cdot$ and an increase in SOD activity results in an increase in $HO\cdot$ (see Figure 7). Because either an increase or a decrease in SOD activity leads to an increase in free radicals, any antioxidant compound has potential therapeutic value for the treatment of ALS.

25 Antioxidants may be provided alone or in combination with SOD polypeptide or gene therapies. Because mutant SOD may cause free radical toxicity for the cells, those antioxidants which do not directly address the imbalance caused by SOD mutant peptides may
30 still be useful therapies or adjunct therapeutics. Useful antioxidants include, for example, vitamin C, vitamin E, lycopene, bilirubin, urate, glutathione, dimercaprol, lutein, ubiquinol-10, dithiothretol, a mercaptan, a sulfa compound, methionine, cysteine or N-

- 30 -

acetyl cysteine. For example, Vitamin E, Vitamin C, lazaroids (Upjohn, Kalamazoo), BHA, BHT, and beta-carotene are all useful therapeutics.

iii) Administration of chelating agents

5 Chelating agents, e.g., desferoxamine, known to chelate transition metals involved in the SOD biochemical pathway may be administered for the treatment of a disease involving a mutant SOD gene, e.g., ALS or FALS. For example, EDTA, EGTA, DETC, BCDA, penicillamine, a
10 metallothionein protein, or a apo-metal binding protein, yeast copper metallothionein, apo-superoxide dismutase, hemoglobin, myoglobin, or plastocyanin.

iv) Administration of monoclonal antibodies to SOD polypeptides

15 Monoclonal antibodies which are specific for the mutant SOD polypeptide may be administered for the treatment of any diagnosed sporadic or familial case of ALS. Polyclonal and monoclonal antibodies which recognize the mutant SOD polypeptide may be obtained
20 using standard techniques known in the art. These antibodies may be subtractive techniques, e.g., by raising polyclonals against mutant SOD, removing those antibodies reactive with normal SOD, and using the remaining antibodies for the preparation of a reagent.
25 The useful monoclonal antibody is an antibody which partially or fully restores the SOD enzymatic activity to the appropriate level in the patient. The desirable antibody may be identified as that antibody which restores SOD levels to within 40% of wild-type levels.
30 Monoclonal antibodies may be tested in vitro by assaying the enzymatic activity of the SOD isolated from a patient in the presence and absence of the monoclonal antibody. Useful antibodies may be used specifically to eliminate

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the activity of the mutant SOD. This approach utilizes monoclonal antibodies specifically reactive with the mutant polypeptide. In the alternative, when the disease symptoms are the result of excess SOD activity,
5 antibodies to both wild-type and mutant SOD polypeptides are therapeutically useful.

v) Administration of anti-sense RNA

Patients diagnosed with a disease in which a causative agent is a mutant SOD gene may be treated by
10 the administration of anti-sense RNA which is complementary to the mutated region of the SOD gene are anti-sense RNA to wild-type SOD. These anti-sense RNA therapeutics may be synthesized using standard techniques to develop anti-sense RNA therapeutics. Anti-sense RNA
15 which recognizes the mutant sequences may be administered for all genetic forms of SOD disease resulting from a SOD mutation. Anti-sense RNA which recognizes wild-type SOD may be administered to reduce levels of SOD enzymatic activity when the disease is a result of excess SOD.

20 *vi) Administration of inhibitors of SOD*

Where the disease is due to an increase in SOD activity, inhibitors of SOD may be administered. For example, peptides derived from wild-type or mutant SOD, non-peptide analogs of SOD, or any small molecule
25 inhibitor of SOD, e.g., diethyldithiol carbamate (Dury et al., *PNAS* 89:9715-9719, 1992) or bathocurprone disulfonic acid may be administered.

vii) Genetic therapy for FALS

**Therapeutic Administration of SOD1, SOD2, or SOD3
30 coding sequences in a Viral Vector.**

Retroviral vectors, or other viral vectors with the appropriate tropism for cells affected by the

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defective SOD gene, e.g. motor neurons involved in ALS, may be used as a gene transfer delivery system for the SOD1, SOD2, or SOD3 genes which encode therapeutic SOD polypeptides. The useful polypeptides to be encoded are described above. Numerous vectors useful for this purpose are generally known (Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis and Anderson, BioTechniques 6:608-614, 1988; Tolstoshev and Anderson, Current Opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; and Miller and Rosman, Biotechniques 7:980-990, 1989; Le Gal La Salle et al., Science 259:988-990, 1993). Retroviral vectors are particularly well developed and have been used in a clinical setting (Rosenberg et al., N. Engl. J. Med 323:370, 1990). In the case of ALS and FALS, poliovirus and herpes virus vectors, which infect neurons, are particularly useful.

The therapeutic SOD polypeptide may also be administered via a retroviral vector which incorporates into the hematopoietic cells, effectively administering the SOD polypeptide systemically in the presence or absence of targeting sequences.

The retroviral constructs, packaging cell lines and delivery systems which may be useful for this purpose include, but are not limited to, one, or a combination of, the following: Moloney murine leukemia viral vector types; self inactivating vectors; double copy vectors; selection marker vectors; and suicide mechanism vectors.

Fragments or derivatives of the Cu/ZnSOD, mSOD, or ecSOD polypeptides may also be administered by retroviral gene transfer therapy or another suitable viral vector system. Useful fragments or derivatives of SOD1, SOD2,

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or SOD3 may be administered by inserting the nucleic acids encoding these fragments or derivatives in place of the complete SOD gene in a gene therapy vector, as described above. Such constructs may be tested using the methods for testing the effects of CuZnSOD, mSOD, or ecSOD on ALS related enzymatic alterations, as described above.

Non viral methods for the therapeutic delivery of nucleic acid encoding Cu/ZnSOD, mSOD, or ecSOD.

10 Nucleic acid encoding Cu/ZnSOD, mSOD, or ecSOD, or a fragments thereof, under the regulation of the wild-type promotor and including the appropriate sequences required for insertion into genomic DNA of the patient, or autonomous replication, may be administered to the patient using the following gene transfer techniques: microinjection (Wolff et al., Science 247:1465, 1990); calcium phosphate transfer (Graham and Van der Eb, Virology 52:456, 1973; Wigler et al., Cell 14:725, 1978; Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413, 1987); lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413, 1987; Ono et al., Neuroscience Lett 117:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger and Papahadjopoulos, Meth. Enz. 101:512, 1983); asialorosanucoid-polylysine conjugation (Wu and Wu, J. Biol. Chem. 263:14621, 1988; Wu et al., J. Biol. Chem. 264:16985, 1989); and electroporation (Neumann et al., EMBO J. 7:841, 1980).

V. Administration of compounds to prevent the onset of symptomatic FALS.

30 In a patient diagnosed to be at risk for FALS any of the above therapies may be administered before the onset of symptomatic ALS.

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VI. Administration of SOD mutant polypeptides for treatment of neoplasms.

Cytotoxic mutant SOD polypeptides can be used to treat neoplasms. Such cytotoxic compounds may be
5 administered using any of the known methods for administering cancer chemotherapeutic agents.

VII. Treatment of sporadic ALS caused by environmental factors.

All therapeutic approaches described herein which
10 alter SOD enzymatic levels or affect reactant or product levels within the SOD biochemical pathway but do not hinge on specific characteristics of the mutant polypeptide, may be used with sporadic ALS which is not the result of a genetic defect.

15 VIII. Diagnosis and Treatment of diseases resulting from mutations in genes encoding non-SOD polypeptides of the SOD biosynthetic pathway.

Diseases caused by deleterious mutations in other polypeptides normally active in the SOD biosynthetic
20 pathway, e.g., catalase, glutathione peroxidase, and nitric oxide synthase, may be diagnosed and treated using the above methods. The above treatments alter SOD activity and the administration of therapies which alter SOD activity will restore the imbalance caused by the
25 perturbations elsewhere in the pathway.

The following examples are to illustrate not limit the invention.

EXAMPLES

I. Identification of the Causative Gene in Familial ALS.

30 Described here is the identification of fourteen different SOD1 missense mutations in sixteen different

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FALS families. Additionally, mutations have been detected by SSCP but not sequenced in five families.

A) Methods

Methods: PCR primers are used in the analysis

5 of SOD1 were:

Exon 2:

Set a 5' ACTCTCTCCAACCTTTGCACTT 3' 5' CCCACCTGCTGTATTATCTCC 3'
Set b 5' TTCAGAACTCTCTCCAACCTT 3' 5' CGTTTAGGGGCTACTCTACTGT 3'

Exon 4:

10 Set a 5' CATATAAGGCATGTTGGAGACT 3' 5' TCTTAGAATTCGCGACTAACAATC
3'
Set b 5' CATCAGCCCTAATCCATCTGA 3' 5' CGCGACTAACAATCAAAGTGA 3'

PCR amplification was performed on Perkin Elmer Cetus or MJ Research thermal cyclers. The program for
15 amplification was as follows: 2 minutes, 95°C initial denaturation; 1 minute each at 95°C, 60°C and 72°C, entrained for 32 cycles; 6 minutes at 72°C final extension. The expected product sizes for exons 2 and 4 are respectively 132 and 214 bp for primer sets a, and
20 207 and 236 for primer sets b. SSCP analysis was performed using MDE^R gels using the manufacturer's recommended protocol (J.T. Baker). Gels containing 5% glycerol were run at room temperature at 4 W for 16 hours. Gels were dried and exposed to film for
25 autoradiography. Sequencing of PCR-amplified exon DNA was performed by purifying the resulting product with Centracon columns (Amicon) and directly sequencing the DNA using Sequenase kits (U.S. Biochemicals).

B) Demonstration of linkage

30 A CA-dinucleotide repeat D21S223 has been identified in cosmid 21-4.25 from the FALS-linked region.

Using the CA-dinucleotide repeat in Cosmid 21-4.25 from the FALS-linked region we have now found that exon 2 of SOD1 can be amplified by the polymerase chain reaction
35 (PCR) from this cosmid. This indicates very close

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proximity of D21S223 and the SOD1 gene. We have confirmed the linkage of D21S223, and therefore SOD1, to FALS: D21S223 produces the highest lod scores yet detected (Table 1). Using the program HOMOG (Ott et al. 5 J. Am. J. Hum. Genetics 28:528-529, 1976; Ott Analysis of Human Genetics 203-216, 1991), we have identified a subset of six of these FALS families in which the disease displays no recombination with D21S223 (Table 1, $z=6.8$ at $\theta=0$) and is likely to be tightly linked to SOD1; nine 10 additional families have also been shown to display significant linkage to the SOD1 region of chromosome 21.

C) Design of PCR primers and SSCP Analysis

To determine if FALS is associated with mutations in the SOD1 gene, PCR primers were designed for two of 15 the five SOD1 exons based on the published sequence for human SOD1 (Levanon et al., EMBO J. 77-84, 1985; Hallewell et al., Superoxide Dismutase in Chemistry, Biology and Medicine 249-256, 1986). These primers were used for PCR amplification of SOD1 exonic DNA from 20 genomic DNA of normal, control individuals and of single FALS-affected individuals from families tightly linked either to SOD1 or neighboring markers on chromosome 21q. The products of the PCR reactions were denatured and separated on a polyacrylamide gel (0.5X MDE, J.T. Baker) 25 for single-strand conformational polymorphism (SSCP) analysis, which detects mobility shifts of single-strand DNA caused by sequence variations (Orita et al., Genomics 5:874-879, 1989). Autoradiograms of these gels revealed shifts in band mobility for 6 of the 15 families linked 30 to the SOD1 region of chromosome 21q. An additional 12 FALS families also revealed anomalous SSCPs; these families were too small for significant linkage analysis. Five of the FALS families are excluded from linkage to chromosome 21q (table 2); none showed abnormal SSCPs. 35 Figure 1a shows the data for SSCP analysis of SOD1 exons

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amplified by PCR from lymphocytes of normal and FALS-affected individuals. Specifically Figure 1A is an autoradiogram showing variations in single-strand conformational banding patterns between normal and FALS DNA for SOD1 exons 2 (top) and 4 (bottom). "N" designates lanes with DNA from normal individuals. The numbers designate lanes with FALS DNA samples and correspond to family numbers in panel B and in Table 3. No band shifts were detected in control DNA samples from normal individuals (140 and 112) respectively for exons 2 and 4 (Table 2).

SSCP analysis was then performed on all available DNA samples from members of families 3, 11, and 192C. In each family, all affected individuals displayed the same band pattern as the originally characterized FALS patient. Additionally, in other family members determined by haplotype analysis to be at risk for FALS, the FALS haplotype cosegregated with the distinctive SSCP variant.

20 D) **Sequencing of SOD1 in affected families**

Direct sequencing of PCR-amplified DNA from exons 2 and 4 was performed for 16 of the 23 FALS families with anomalous SSCP bands. In each instance, there was heterozygosity in the DNA sequence indicative of one normal and one abnormal chromosome. As summarized in Table 3, we identified single base pair changes in all 13 of these families. These 13 mutations predict eleven distinct amino acid substitutions. Two different amino acid substitutions were detected in each of two codons (41 and 93; Table 3). In each of two codons (37 and 113), two apparently unrelated families have the same mutation. The same mutation in codon 93 was detected independently in two branches of the same family (designated 3 and 3-192C, Table 3).

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Representative data is shown in Figure 1B. Figure 1B are sequence analysis of SOD1 exons amplified by PCR from genomic DNA from lymphocytes of normal and FALS-affected individuals specifically shown in sequence analysis of SOD1 exons 2 and 4 in genomic FALS DNA; the numbers above each sequence ladder identify the affected family, corresponding to family numbers in panel A and in Table 3. The vertically oriented sequence to the left of each ladder designates nine base pairs of sequence including the mutation, indicated by a double base pair, with the wild-type base on the left; the arrowhead denotes the position within the sequence showing heterozygosity as indicated by two (normal and FALS) base pairs. Sequence ladders read 5'→3' from top to bottom.

Nine of the eleven sequence changes alter recognition sites for restriction enzymes (Table 3). For example, in family 11, a new MaeIII site (GTNAC) results from the C→G transition (normal sequence CTGAC). To confirm these sequence changes, we digested PCR products of the corresponding exons with the appropriate restriction enzymes. MaeIII digestion of the exon 2 PCR product for affected members of family 11 produced three bands on a denaturing acrylamide gel (Sequagel 6 (National Diagnostics)): a 132 bp full length product and products of 72 and 60 bp, the fragment sizes expected given the extra MaeIII site in the mutant DNA. These fragments were not detected in normal, control DNA samples from 70 unrelated members of our ALS families or from 73 unrelated members of reference pedigrees in the Centre d'Etude du Polymorphisme Humain (CEPH, (Dausset et al., Genomics 6:575-577, 1990)). The base pair changes in families 3, 118, 130 and 684C introduce other novel restriction sites; additionally, in families 33, 36, 130, and 684C the single-base changes eliminate restriction sites normally present in SOD1.

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These changes occur in all 13 of these families. These 13 mutations predict eleven distinct amino acid substitutions. Two different amino acid substitutions were detected in each of two codons (41 and 93; Table 3).

5 In each of two codons (37 and 113), two apparently unrelated families have the same mutation. The same mutation in codon 93 was detected independently in two branches of the same family (designated 3 and 3-193C, Table 3).

10 These studies identify eleven single amino acid changes in SOD1 based upon the genomic DNA sequences of members of thirteen different FALS families. These changes were not detected in more than 100 chromosomes from normal individuals and thus it may be concluded that
15 these mutations are not simply normal allelic variants. Instead, these mutations occur in tight association with FALS. It is concluded that the mutations identified herein in the SOD1 gene are the mutations which cause FALS.

20 II. Identification of an ala 4 to val mutation

We screened exon 1 of SOD1 in DNA from 167 FALS pedigrees using single-strand conformational polymorphism (SSCP) analysis (Orita et al. (1989) *Proc. Natl. Acad. Sci. USA* 86, 2766-2770; Orita et al. (1989) *Genomics* 5,
25 874-879) (Figure 8A). Identical variant exon 1 SSCP patterns were detected in 14/172 FALS families (designated as families # 45, 103, 104, 114, 125, 127, 156, 160, 176, 189, 221, 224, 228, and 233). No other variant SSCP patterns were seen in the DNA encoding exon
30 1 in the other 159 FALS families or in 130 patients with sporadic ALS. A different SSCP band shift was observed in a single normal individual in a total of 116 assayed, but determination of the DNA sequence revealed that this shift was not caused by a change in the coding region for

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exon 1. As shown in Figure 8B, DNA sequence analysis revealed a C-to-T transition at base pair 14 of SOD1 exon 1 in affected individuals with abnormal SSCPs. This missense mutation predicts a valine for alanine substitution at codon 4. The C-to-T mutation eliminates restriction enzyme sites for *Mwo* I (GCN₇GC) and *Hae* III (GGCC). Thus, when the mutation is present, *Hae* III digestion of the above exon 1 PCR product produces fragments of 38, 49, and 95 base pairs; by comparison, without the mutation, the expected fragments are 24, 25, 38, and 95 base pairs. Similarly, when the mutation is present, *Mwo* I does not digest the 182 bp exon 1 PCR product whereas without the mutation, digestion with this enzyme produces fragments of 71 and 111 bp. Ala 4 is highly conserved in the SOD1 enzyme of many species (Levanon et al. (1985) *EMBO J.* 4, 77-84; Cannon et al. (1987) *Isozymes Curr. Top. Biol. Med. Res.* 14, 73-81; Hjalmarsson et al. (1987) *Proc. Natl. Acad. Sci. USA* 84, 6340-6344; Perl-Treves et al. (1988) *Plant Mol. Biol.* 11, 609-623; Montesano et al. (1989) *Eur. J. Biochem.* 186, 421-426; Seto et al. (1989) *Gene* 75, 85-92; Chary et al. (1990) *J. Biol. Chem.* 265, 18961-18967; Benedetto et al. (1991) *Gene* 99, 191-195; Hallewell et al. (1991) *Biochem. Biophys. Res. Commun.* 181, 474-480) (8C) consistent with the hypothesis that this site is important for enzyme function. The restriction enzyme site changes associated with the SOD1 Ala4 to Val mutation are summarized in Table 4.

The ala 4 to val mutation in the SOD1 gene is the most commonly identified mutation associated with FALS. We have identified 14 apparently independent occurrences of this mutation. This represents 14/36 or 38.9% of our FALS families with identified SOD1 mutations. Deng et al. ((1993) *Science* 261, 1047-1051) reported an additional eight occurrences in 17 FALS families with

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identified SOD1 mutations. By comparison, the next most frequent mutations we have identified (Rosen et al.

(1993) Nature 362, 59-62), gly 37 to arg and ile 113 to thr, have each been detected in two independent families.

5 The ala 4 to val mutation is associated with a consistently severe clinical phenotype in terms of duration of survival after disease onset (Table 5). Patients with the ala 4 to val mutation survive only an average of 1.2 years after disease onset, as compared to
10 2.5 years for the average survival of all other FALS patents. By contrast, the age of onset in the exon 1 families is similar to that in other FALS patients. Thus, the ala 4 to val exon 1 mutation underlies an aggressive form of ALS.

15 Exon 1 PCR primers used for these SSCP studies were:

5'ATAAAGTAGTCGCGGAGACGG-3' (SEQ ID NO: 18) and

5'-GCCTTCTGCTCGAAATTGATG-3' (SEQ ID NO: 19). The

expected product size is 182 bp. PCR amplification in

20 Perkin Elmer Cetus or MJ Research thermal cyclers entailed initial denaturation (95°C, 2 min) and 32 cycles of 1 minute each at 95°C, 60°C, 72°C and then 6 minutes at 72°C for final extension. SSCP analysis was performed using MDE gels (J.T. Baker) with the manufacturer's
25 recommended protocol. Sequencing of PCR-amplified DNA involved further purification of the products on Centricon columns (Amicon) and direct sequencing with a Sequenase kit (U.S. Biochemicals) using the above primers. Sequencing gels were prepared using Sequagel-6
30 (National Diagnostics) according to the manufacturer's recommended protocol.

III. Expression of SOD1 in ala to val individuals

The observation that SOD1 mutations are associated with FALS suggests that SOD1 may play an important role
35 in motor neuron survival. Based on this hypothesis, we

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examined the expression of SOD1 in central nervous system tissues from normal individuals and three patients carrying the exon 1 ala 4 -> val mutation. In normal spinal cord, *in situ* hybridization with a 51-mer probe
5 (SEQ ID NO: 20) from coding sequence unique to SOD1 revealed abundant expression of the mRNA for this protein in large neurons in the anterior quadrant (Figure 9).

Consistent with this observation, immunostaining of normal spinal cord with a monoclonal antibody to human
10 SOD1 showed diffuse expression of the protein throughout the grey matter of the spinal cord and marked prominence of expression in large neurons including motor neurons, Clarke's column neurons, and neurons within the posterior horn (Figure 10 and data not shown). This staining
15 appeared throughout the cytoplasm and nucleus and extended into the processes of the large neurons. We also observed prominent staining in large neurons in the motor cortex, including Betz cells (data not shown).

In two individuals with the ala 4 to val mutation,
20 pronounced immunostaining of SOD1 in large neurons in Clarke's column and posterior horn was evident as in normal individuals (data not shown). However, in association with pre-terminal motor neuron deterioration, reduced staining was seen in the anterior horn of the
25 spinal cord (Figure 11), although staining with cresyl violet and anti-neurofilament antibody indicated the presence of a few residual motor neurons.

Quantitation indicated that the density of anterior horn cells as assessed by cresyl violet (CV),
30 anti-neurofilament (SMI-32), and anti-SOD1 staining in tissue from these two patients with the exon 1 mutation was decreased by about 80% as compared to four non-FALS controls. In the FALS patients, the number of motor neurons staining with SOD1 antibody was only about two-

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thirds of the number stained by either cresyl violet or anti-neurofilament antibody.

To understand the functional consequence of the ala 4 to val mutation, we studied SOD1 enzyme activity in lysates of red blood cells from two FALS-affected females carrying this mutation. Red blood cell lysates from both sexes of two other groups were also studied: normal individuals with no known neurological diseases (18 males and 13 females) and individuals with sporadic ALS (SALS; 9 males and 6 females). The mean SOD1 activities for control females and males did not differ significantly ($p=0.054$ by ANOVA); in similar, more extensive analyses by others, no significant differences in RBC lysate SOD1 activity have been observed between males and females (S. Marklund, personal communication). We have therefore compared the female FALS SOD1 activities to the combined male and female SOD1 activities for the two other groups. The two female FALS patients with the ala 4 to val mutation showed statistically significant reductions in SOD1 enzymatic activity per mg total protein (Table 6) compared to specimens from 31 normal controls ($p=0.014$) and 15 SALS patients ($p=0.013$).

We also examined SOD1 activity in lymphoblastoid cell line established from a patient with this exon 1 mutation. To determine the effects of gene dosage of SOD1 on corresponding enzymatic activity, we examined lymphoblastoid cell lines from individuals who were mono-, di- and trisomic for chromosome 21. In these cell lines, the relative SOD1 enzyme activity levels for the mono-, di-, and trisomic cell lines were approximately 1:2:3, respectively (Table 7). The cell line from the patient with the ala 4 to val mutation in SOD1 had a level of enzymatic activity roughly comparable to that in the monosomy 21 patient. Using the same lines, we performed Western immunoblots to gauge the relative

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levels of SOD1 protein. The observed overall protein levels of SOD1 in the mono-, di- and trisomic lines were roughly 1:2:3 (Table 7). The level of SOD1 protein in the exon 1 line was approximately half the normal level and was comparable to the monosomy 21 level. Thus, SOD1 activity per unit SOD1 protein was approximately normal in the lymphoblastoid line carrying the ala 4 to val mutation, although total SOD1 enzymatic and protein levels were only about one-half of normal.

Using cytosol from frontal cortex (Brodmann area 11), we determined SOD activities in three FALS patients with the ala 4 to val exon 1 mutation and 12 normal controls. Among the controls there was no significant sex difference, correlation of activity with age ($r = -0.103$), or postmortem interval ($r = -0.100$). SOD activity was reduced by 43.2% in the ala 4 to val FALS patients relative to the controls ($p = 0.015$; Table 8). In a comparable brain sample from a single, age-matched FALS patient without a known SOD1 mutation, the SOD1 activity was 376 SOD₅₂₅ U/mg, a value that is not significantly different from that of controls ($p = 0.87$).

A. SOD1 Enzymatic Activity

Three methods were used to determine SOD1 activity levels. For the brain tissue samples we quantified inhibition of spontaneous oxidation of epinephrine as a measure of SOD1 activity (Misra et al. (1972) *J. Biol. Chem.* 247, 3170-3175). The brain SOD activity was inhibited by 70-80% with 5 mM KCN. For the lymphoblastoid cell lines (Anderson et al. (1984) *In Vitro* 20, 856-858), we used a commercial assay (Randox Laboratories, Antrim, Northern Ireland) that employs xanthine and xanthine oxidase to generate superoxide radicals that interact with 2-(4-indophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. In this assay, SOD activity, detected

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spectrophotometrically at 505 nm, is measured by the degree of inhibition of this reaction. Erythrocyte lysates were prepared by chloroform:ethanol extraction (Winterbourn et al. (1975) *J. Lab. Clin. Med.* 85, 337-341). For the red cell lysates, two assays were used: the epinephrine autoxidation assay (Misra et al. (1972) *J. Biol. Chem.* 247, 3170-3175) and SOD-525 commercial assay kit (Bioxytech, Cedex, France) in which SOD1 initiates a reaction with a proprietary chromophore. One unit/mg in the SOD-525 assay corresponds to 369.8 units/mg by the epinephrine autoxidation assay. Differences between test groups were analyzed by analysis of variance (ANOVA) followed by Fisher's PLSD post-hoc test (Zar, J.H. (1974) *Biostatistical Analysis*. Prentice Hall, Englewood Cliffs, N.J.).

B. Western Immunoblotting of SODS Polypeptide

Proteins from crude cell extracts were separated by discontinuous SDS polyacrylamide gel electrophoresis (Laemmli, U.K. (1970) *Nature* 227, 680-685) using 12% (w/v) gels. The separated proteins were transferred by electroelution onto nitrocellulose as described (Towbin et al. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4350-4354). The nitrocellulose membrane was blocked with 5% non-fat dried milk in a Tris-buffered saline (TBS) for 30 minutes, followed by incubation in buffer A (1% non-fat dried milk, 0.2% Triton X-100 in TBS) containing the anti-(human SOD1) antibody for 90 minutes. The anti-(human SOD1) antibody was used at 1000:1 dilution. The membrane was washed in buffer A thrice for 5 minutes, then incubated for 1 hour in buffer A containing anti-mouse IgG-horseradish peroxidase conjugate (BioRad). The membrane was then washed in buffer A twice for 5 minutes, followed by TBS for 5 minutes. The membrane was incubated in ECL reagents (Amersham) for 1 minute, sealed in plastic wrap, and placed on X-ray film for 15-120

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seconds. Films were scanned using a Helena Labs Quick Scan densitometer. Several exposures were used and several protein concentrations analyzed to permit integration within the linear range of film exposure.

5 C. In Situ Hybridization

Human spinal cords were removed one hour post-mortem, frozen on dry ice and kept at -80°C until sectioning. Later 12 µm thick frozen sections were cut using a cryostat and fixed for 5 minutes with 4%
10 paraformaldehyde in phosphate buffered saline (PBS) pH 7.4, then treated with 0.25% acetic anhydride in 0.1 M triethanolamine, 0.9% NaCl pH 8.0 for 10 minutes. Following dehydration in increasing concentrations of ethanol, lipids were extracted from the sections with
15 chloroform and air dried. Hybridization reactions were performed in 50% formamide, 4X, SSC (1X SSC=0.15 M NaCl, 0.15 M sodium citrate pH 7.2), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 500 µg/ml sheared single-stranded salmon sperm DNA, 250 µg/ml
20 yeast tRNA, 10% dextran sulphate and 50 mM dithiothreitol for 16 hours at 37°C. Slides were rinsed in 1X SSC and washed for four 15 minute intervals in 2X SSC, 50% formamide at 40°C, followed by two 60 minute rinses in 1X SSC at 25°C. The sections were briefly rinsed in
25 distilled water, 70% ethanol, and 95% ethanol and then air dried for autoradiography. The sections were coated with Kodak NTB3 nuclear track emulsion and developed after 2 weeks. Toluidine blue was used as a background stain. The sequence of the 51-mer oligonucleotide,
30 derived from the human SOD1 gene, is
5'ATGCAGGCCTTCAGTCAGTCCTTTAATGCTTCCCCACACCTTCACTGGTCC3'
(SEQ ID NO: 20). Hybridization with the sense probe did not result in any specific labeling.

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D. Immunohistochemistry

Brains were blocked freshly at the time of autopsy and flash frozen in liquid nitrogen or fixed in periodate lysine paraformaldehyde for 48 hours at 4°C, followed by
5 cryoprotection in 20% glycerol, 2% dimethylsulfoxide in phosphate buffer and sectioning at 50 μ m on a sledge microtome. Sections were washed in cold 0.1M PBS pH 7.3, incubated in 10% normal goat serum in PBS, and placed in dilute monoclonal antibody specific for human Cu/Zn
10 superoxide dismutase (Sigma, clone SD-G6, 1:20-1:100) or nonphosphorylated neurofilament (SMI 32, Sternberger Monoclonals, 1:2000) in 5% normal goat serum and 0.3% Triton X-100 overnight at room temperature on a rocker. The next day sections were washed several times in PBS
15 and incubated in 0.05% diaminobenzidine tetrahydrochloride in 0.005% hydrogen peroxide in Tris-HCl buffer pH 7.5 and monitored by intermittent microscopic examination. The peroxidase reaction was terminated by washes in PBS. Sections were mounted, air
20 dried, and coverslipped. Other sections were stained with cresyl violet. The time to autopsy and the state of autopsied tissue did not differ between FALS and control samples; thus, assessment of cresyl violet-stained sections from non-motor regions (sensory cortex in brain;
25 dorsal horn in spinal cord) revealed no obvious differences between normal and FALS tissues.

All motor neurons in each section stained with cresyl violet, anti-SOD-1, and anti-neurofilament were counted and their density expressed per anterior horn.
30 Quantitative data were obtained from spinal cords and two FALS patients with the exon 1 mutation and six normal individuals. The number of motor neurons stained with cresyl violet (CV) and SMI32 and SOD1 antibodies were counted in each anterior horn in each section. Multiple
35 sections were counted in each case. From these sections,

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the average number of motor neurons per anterior horn per section was calculated. Statistical analysis was performed using unpaired t-test and one factor ANOVA.

E. Mechanism of Mutant SOD1 Pathogenesis

5 The above-mentioned results show that a missense mutation in exon 1 of the SOD1 gene is found in association with FALS. This ala4 to val substitution is the most common single SOD1 mutation we have detected. We have not detected any SOD1 mutations in over 100
10 controls and 100 sporadic ALS (SALS) patients. The association of SOD1 mutations with FALS suggests that wild-type SOD is important for the long-term function and viability of neurons, particularly motor neurons. It is possible that normal SOD expression is required in non-
15 neuronal cells, particularly glial cells, for example astrocytes; microglia; interneurons or immune system cells.

 The SOD1 Ala4 to val mutation reduced total activity by 50% compared to normal controls. The exon 1
20 SOD1 ala4 to val gene mutation may be an antimorphic or dominant-negative gene mutation; a gain-of-function or neomorphic gene mutation; or a loss-of-function or a haplo-insufficient gene mutation. Each of these possibilities may result in a mutant SOD1 protein that
25 does not normally remove oxygen free radicals, or produces uncommon, toxic oxygen species. It is also possible that reduction or loss of normal SOD1 function leads to a FALS phenotype via abnormal copper binding. For example, if the copper-binding site of SOD1 (a Cu/Zn
30 enzyme) has reduced metal affinity, copper ion levels may elevate to toxic levels. Copper is a known neurotoxin (Scheinberg, (1988) In: Bondy et al. (eds) *Metal Neurotoxicity*, CRC Press, Boca Raton, Florida, pp. 55-60) and can participate in a variety of redox reactions to
35 generate toxic-free radicals (Halliwell et al., (1992)

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In: Scandalios (ed) *Molecular Biology of Free Radical Scavenging Systems*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 47-67), administration of copper chelating agents may prove efficacious in
5 preventing or reducing the severity of FALS, SALS, or any other neurodegenerative diseases described herein.

III. Additional Exon 3 and Exon 4 Mutations in SOD1

Single-strand, conformational polymorphism (SSCP) analysis of SOD1 exon 4 revealed an altered banding
10 pattern for an asymptomatic individual in family 202 whose identical twin had previously died from ALS. Additionally, an altered SSCP pattern was observed for a FALS patient in family 212. These variant SSCP bands were not seen in DNA samples from 168 other FALS
15 patients, 169 sporadic ALS patients, and 100 normal controls.

DNA sequence analysis of exon 4 for these FALS patients revealed single base pair substitutions at codon 93 (GGT to GAT) in family 212 and codon 112 (ATC to ACC)
20 in family 202 (Figure 12). These changes create glycine 93 to aspartic acid and isoleucine 112 to threonine missense mutations in SOD1 in these FALS patients. The codon 112 mutation leads to a loss of an Sfa NI restriction enzyme site normally present in the wild-type
25 SOD1 sequence of exon 4. Both mutations are in highly conserved amino acids.

We have also found a polymorphism in intron 3 of the SOD1 gene. SSCP analysis revealed its presence in 7 of the 157 FALS samples and 11 of 100 normal controls.
30 DNA sequence analysis of the proximal segment of intron 3 revealed an A-to-C substitution 34 base pairs downstream of exon 3, counting from the G in the GT splice donor site (Figure 13). Allele frequencies for the two forms of the intron 3 polymorphism are 94.5% for the A-

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containing allele and 5.5% for the C-containing allele. The heterozygosity (Ott, *Analysis of Human Genetics*, (1991) Johns Hopkins University Press, Baltimore, pp. 25-27) of these two allele polymorphism is 0.1. This A-to-C transversion in intron 3 creates novel sites for Hae II, Hha I and Hin PI restriction enzymes. The presence of the polymorphism in intron 3 was confirmed for all SSCP variants by restriction enzyme digestion of the PCR product (data not shown). The DNA sequence we have obtained in this region (Figure 13) differs slightly from that previously published (Levanon et al., (1985) *EMBO J.* 4, 77-84; Hallewell et al., (1986) in *Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine*, ed. Rotilio, pp. 249-256).

We have performed SOD1 assays on red cell lysates from individuals with these mutations (Winterbourn et al., (1975) *J. Lab. Clin. Med.* 85, 337-341). In the individual with the G112T mutation, SOD1 activity was 2.8 U/mg total protein, which is less than the 10% confidence limits for the mean SOD1 activity (4.2 ± 1.0 U/mg) of 31 individuals with no neurological diseases ($P(Z=1.4)=0.081$) (Zar et al., (1984) in *Biostatistical Analysis*, Prentice-Hall, Englewood, NJ, 2nd edition, pp. 83-86). In four individuals with the G93A mutation, SOD1 activity was 3.6 ± 0.9 U/mg; which does not differ significantly from the 31 controls.

We note that survival in the G93A family (15 years in the index case) may be longer than in our other FALS families (mean 2.5 years, 99.9% confidence interval 8.8 years); by contrast, mean survival in two individuals with the G112T mutation was 0.9 ± 0.1 years. These findings are consistent with the observation of Aoki and colleagues (*Nature Genetics* (1993) 5, 102-103) that FALS mutations producing the mildest loss of SOD1 function may cause the mildest forms of the disease.

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These results are similar to those found with exon 1 SOD1 gene mutations, to the extent that a minor loss of SOD1 function results in a dominant phenotype. It is possible that some models (see above) which explain the phenotype of the Ala4→ Val gene mutation in the SOD1 exon 1 may also apply to these exon 3 and exon 4 gene mutations.

IV. Effective therapies for preventing or reducing the severity of ALS

Each therapy may be used alone or in combination with one or more therapies as disclosed herein. The following formulations may be administered by any means described above.

A) Administration of an Antioxidant Formulation:

One or more antioxidants may be administered in order to prevent or reduce the level of reactive free radicals, particularly oxygen free radicals; in a cell, particularly a motor neuron. For example, the diet may be supplemented with one or more of an antioxidant, preferably, vitamin C (ascorbate), vitamin E (alpha tocopherol), beta-carotene, lycopene, bilirubin, urate, glutathione, dimercaprol, lutein, ubiquinol-10, dithiothretol, mercaptans, preferably mercaptoethanol, sulfa compounds (i.e., the sulfonamides), for example, sulfa antibiotics such as N-sulfanilylbenzamide, amino acids or derivatives thereof, such as methionine, cysteine, or N-acetyl cysteine. Methods of administration and preferred dosages have been disclosed by Greengard (P. Greengard, in The Pharmacological Basis of Therapeutics, Goodman, L.S. and Gilman, A. 5ed. (1975); herein incorporated by reference). For compounds and methods of administration not disclosed in Greengard (supra), between 0.1mg to 100mg inclusive can be

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administered per day to an adult. Administration of an antioxidant formulation may be by any method disclosed herein. The actual dosage will depend on a number of factors, including the health of the individual patient
5 and the progression of ALS. An antioxidant, as used herein, is any chemical capable of scavenging free radicals formed in a biological reaction.

B) Administration of a Drug Formulation: One or more drugs may be administered in order to prevent or
10 reduce the level of reactive free radicals, particularly oxygen free radicals; in a cell, particularly a motor neuron. Drugs that are capable of inhibiting enzymatic systems which are associated with free radical production include deprenyl (a monoamine oxidase inhibitor) and the
15 lazeroid compounds (inhibitors of lipid peroxidation). Dosages and methods of administering deprenyl have been disclosed (Physician's Desk Reference 47 pg. 2351 (1993)). Clinical use of the lazeroid compounds has been discussed (J. M. McCall et al. Acta Anaesthesiol. Belg.
20 38, 417 (1987)). Administration of a drug formulation may be by any method disclosed herein. The actual dosage will depend on a number of factors, including the health of the individual patient and the progression of ALS.

C) Administration of an Enzyme Formulation: Direct
25 intrathecal administration of one or more enzymes capable of scavenging free radicals can be performed in order to prevent or reduce the level of free radicals in a cell, particularly a motor neuron. For example, SOD-1 may be administered alone or in combination with one or more
30 enzymes capable of scavenging free radicals, such as SOD-2, SOD-3, or catalase. The actual dosage of enzyme will depend on a number of factors, including the health of the individual patient and the progressive of ALS. Generally, 0.01 ug to 100ug will be administered in a

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pharmaceutically acceptable formulation as described herein.

D) Administration of A SOD-1 Transgene:

Functional copies of a SOD-1 gene into a cell, preferably
5 a motor neuron cell, may be accomplished by delivery
systems, for example viral vectors comprising portions of
polio or herpes virus genomic DNA, retroviruses; or
liposomes comprising targeting proteins or lipids.
Methods for the therapeutic delivery of SOD-1 or other
10 SOD genes are described herein.

E) Administration of Oligonucleotide Formulations:

DNA oligonucleotides capable of binding with mutant SOD-1
mRNA may be designed. Preferably, the DNA oligonucleotide
is at least 15 nucleotides, more preferably at least 25
15 nucleotides and most preferably at least 50 nucleotides
in length. The DNA oligonucleotide is substantially
homologous to the wild-type SOD-1 gene or is
substantially homologous to any mutant SOD-1 gene
described herein. The binding of one or more DNA
20 oligonucleotides to a mutant SOD-1 mRNA will prevent
protein translation of the mutant mRNA. Methods of
administering a DNA oligonucleotide formulation have been
described. The actual dosage of a DNA oligonucleotide
formulation will depend on a number of factors, including
25 the health of the individual patient and the progressive
of ALS. Generally, 0.001 ug to 10ug will be administered
in a pharmaceutically acceptable formulation as described
herein.

F) Administration of a Metal Chelating Agent:

30 Mutant SOD-1 function may modulate metal levels within a
cell, for example, copper and/or zinc levels within a
motor neuron. The administration of one or more metal
chelating agents, preferably agents capable of chelating
copper and/or zinc, may prevent or reduce the severity of

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ALS. Examples of chelating agents include EDTA (ethylenediamine tetraacetic acid), EGTA (ethleneglycol bis-beta-aminoethyl ether) N',N'-tetracetic acid), desferroxamine, DETC (diethyl-dithiocarbamate), BCDA
5 (bathocurprone disulfonic acid), and penicillamine, and tetracycline. (Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis and Anderson, BioTechniques 6:608-614, 1988; Tolstoshev and Anderson, Current Opinion in Biotechnology 1:55-61, 1990;
10 Sharp, The Lancet 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; and Miller and Rosman, Biotechniques 7:980-990, 1989; Le Gal La Salle et al.,
15 Science 259:988-990, 1993). A metal chelating agent may also be a polypeptide, for example, a metallothionein protein, preferably yeast copper metallothionein; plastocyanin, apo-superoxide dismutase, or other apo-metal binding proteins. The administration of chelating
20 agents and preferred dosages have been disclosed in Goodman and Gilman (The Pharmacological Basis of Therapeutics, 5Ed. Chapter 11, (1975); Physician's Desk Reference 47, pg 892 (1993)). For compounds and methods of administration not disclosed (supra), between 0.1mg to
25 100mg inclusive of a chemical chelating agent or between 0.01ug to 100ug of a polypeptide chelating agent can be administered per day to an adult. The actual dosage will depend on a number of factors, including the health of the individual patient and the progression of ALS.
30 Administration of a metal chelating agent may be in the form of a therapeutic formulation as disclosed herein. The route of administration may be any route disclosed herein.

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G) Administration of Peroxide-Reducing

Polypeptides: A key function of SOD is to detoxify the superoxide free radical (i.e., O_2^-), converting it to hydrogen peroxide (i.e., H_2O_2). In turn, hydrogen peroxide is converted to water by either catalase or glutathione peroxidase. Hydrogen peroxide itself can generate free radical more harmful than superoxide, for example the hydroxyl radical (i.e., OH^-). Therefore, peroxide-reducing polypeptides capable of lowering the level of hydrogen peroxide in a cell, preferably a motor neuron, can be effective in preventing or reducing the severity of ALS. One or more peroxide-reducing polypeptides may be administered to a patient in order to prevent or reduce the severity of ALS. Examples of peroxide-reducing polypeptides include catalase, glutathione peroxidase, selenium dependent glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, cytochrome c peroxidase, for example, the cytochrome c peroxidase from yeast mitochondria, ascorbate peroxidase, for example, ascorbate peroxidase from plants, NAD(P)H peroxidase, for example, from bacteria, guaiacol peroxidase, for example, from horseradish, ceruloplasmin, a polypeptide capable of exhibiting ferroxidase activity, the reductases, for example glutathione reductase, monodehydroascorbate reductase, or dehydroascorbate reductase.

A peroxide-reducing polypeptide may also be a "sacrificial" polypeptide which is capable of being reduced. The reduction of a sacrificial polypeptide would prevent or reduce reduction of key cellular polypeptides. Examples of sacrificial polypeptides include albumin, transferrin, ferritin, or any protein comprising thiol groups. Any method of administration disclosed herein may be used to administer a peroxide-reducing polypeptide. Preferably, the peroxide-reducing polypeptide is administered intrathecally in a dose range

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of between 0.01ug to 100ug per day to an adult. The actual dosage will depend on a number of factors, including the health of the individual patient and the progression of ALS.

5 Further embodiments are within the following claims.

Table 1

Linkage Analysis of FALS Pedigrees with
SOD1 Marker D21S223 (DB1)

Theta	Lod Score						
	<u>0</u>	<u>5</u>	<u>10</u>	<u>15</u>	<u>20</u>	<u>25</u>	<u>30</u>
All families ¹	-∞	3.83	4.25	4.05	3.55	2.87	2.11
21-linked families ²	6.80	6.05	5.26	4.44	3.61	2.77	1.93

1 - Data are tabulated from an analysis of 12 families in the Boston arm of the FALS collaborative study (7,11).

2 - A subset of six families with a high (>80%) posterior probability of linkage to D21S223 as defined using the program HOMOG (12). Some of the eleven mutations described in Table 3 were detected in DNA from members of FALS families too small for significant linkage analysis. Such families could not be included in the linkage data summarized in this Table.

Table 2

17 Single-Strand Conformational Polymorphisms
in Exons 2 and 4 of SOD1 in FALS DNA¹

	<u>All FALS</u> ²	<u>FALS-21</u> ³	<u>Control</u> ⁴
EXON 2			
Normal	148	15 ⁵	140
Variant	76	1	0 ⁷
EXON 4			
Normal	150	15	112
Variant	11 ⁸	5	0

- 1 See legend for Figure 1 for Methods.
- 2 Familial ALS
- 3 Subset of families linked by HMOG (12) to SOD1 (Boston) or adjacent markers (Chicago).
- 4 DNA samples from normal individuals unrelated to members of FALS families.
- 5 Six from Boston and nine from Chicago FALS pedigrees.
- 6 Includes four, two and one samples respectively from Boston, Chicago and Montreal FALS pedigrees.
- 7 12 control DNA samples revealed weak and somewhat variable SSCPs; all were normal by sequence analysis.
- 8 Includes five, five and one samples respectively from Boston, Chicago and Montreal FALS pedigrees.

TABLE 3A Base Pair Changes in SOD-1

family	exon	amino acid	codon	new codon	new amino acid
103	1	ala 4	GCC	GTC	val
104	1	ala 4	GCC	GTC	val
114	1	ala 4	GCC	GTC	val
127	1	ala 4	GCC	GTC	val
18	2	gly 37	GGA	AGA	arg
594C	2	gly 37	GGA	AGA	arg
11	2	leu 38	CTG	GTG	val
33	2	gly 41	GCC	AGC	ser
36	2	gly 41	GCC	GAC	asp
220C	2	his 43	CAT	CGT	arg
9967C	4	gly 85	GGC	CGC	arg
57	4	gly 93	GGT	TGT	cys
3	4	gly 93	GGT	GCT	ala
3-192C	4	gly 93	GGT	GCT	ala
37	4	glu 100	GAA	GGA	gly
684C	4	glu 100	GAA	GGA	gly
118	4	leu 106	CTC	GTC	val
130	4	ile 113	ATT	ACT	thr
385C	4	ile 113	ATT	ACT	thr
78	5	leu 144	TTG	TCG	ser
113	5	ala 145	GCT	ACT	thr

TABLE 3B
SOD1 Mutations in Familial Amyotrophic Lateral Sclerosis

Family	Base Pair Change	Amino Acid Change	Predicted Restriction Enzyme Change	PCR Product (bp)		Restriction Fragments (bp)	
				1		Normal	FAIS-Specific ²
18, 594C	GGA -> AGA	gly 37 -> arg					
11	CIG -> GIG	leu 38 -> val	+ MaeIII ^{3,4}	132	132	132	72, 60
33	GGC -> AGC	gly 41 -> ser	- HaeIII ⁵ - StuI - Eco57I	132 132 132	83, 49 83, 49 97, 35	132 132 132	132 132 132
36	GGC -> GAC	gly 41 -> asp	- HaeIII ^{3,5}	132	83, 49	132	132
220C	CAT -> CGT	his 43 -> arg	- NlaIII	132	87, 30, 9, 6	96	96

xon 2

1

TABLE 3C
SOD1 Mutations in Familial Amyotrophic Lateral Sclerosis

Exon 4		GCC -> GGC	gly 85 -> arg	+ HlnPI + HhaI + FspI	214 214 214	192, 22 192, 22 193, 21
9967C						
57		GGT -> TGT	gly 93 -> cys	214		
3, 3-192C ⁶		GGT -> GGT	gly 93 -> ala	214	112, 100	72, 40
684C		GTT -> GGA	glu 100 -> gly	214 88, 68, 58	148, 66 165	
118		CTC -> GTC	leu 106 -> val	214	120, 89, 5	83, 6
110, 385C		ATT -> AGT	ile 113 -> thr	214 214 214	124, 90 116, 98 104, 57, 53	113, 11 214 161

⁶based on primer sets a (see Figure Legend).

Other size products are predicted but not listed because they are not FALS-specific.

Predicted changes in indicated restriction fragments have been confirmed in genomic DNA from FALS patients.

The FALS MaelII, DdeI and BsrI fragments were not seen in DNA respectively from 143, 73 and 73 control individuals.

All 73 DNA samples from normal controls showed the expected MaelII restriction site.

The suffix C denotes families analyzed in Chicago. Families 3 and 3-192C are different branches of the same pedigree.

TABLE 4 Restriction Site Changes Associated with SOD1 Ala 4 to Val Mutation

Base-pair change	Predicted			Restriction fragments (bp)	
	Amino acid change	Restriction enzyme site lost	PCR product (bp) ¹	Normal	FALS
GCC->GIC	ala 4 -> val	Hae III (GGCC)	182	95, 38, 25, 24	95, 49, 38
		Mwo I (GCN ₇ GC)	182	111, 71	182

¹size of PCR product using primers described in Methodology

TABLE 5

**Age of onset and survival: ala 4 to val mutation vs other
FALS pedigrees**

	A4V FALS	All Other FALS
Survival		
mean	1.2	2.5
n	24	214
std. dev.	0.8	1.9
Age of Onset		
mean	51.5	49.1
n	24	250
std. dev.	11.2	13.7

For survival, the difference between the two groups is significant at $p < 0.001$ (student's t-test); the difference between the ages of onset is not significant. A4V designates the alanine 4 to valine substitution.

TABLE 6**Red blood cell lysate SOD1 enzymatic activity¹**

	A4V		
	FALS	SALS ²	Controls
Females			
mean	2.6	5.0	3.8
n	2	5	13
std. dev.	1.0	0.9	0.7
Males			
mean		4.7	4.5
n	0	10	18
std. dev.		1.1	1.1
Total			
mean	2.6	4.8	4.5
n	2	15	31
std. dev.	1.0	1.1	1.0

¹ SOD units/mg total protein ² sporadic ALS

The p values for A4V FALS vs SALS and controls are 0.013 and 0.014, respectively (Student's t-test). For SALS vs control, the p value is 0.35.

TABLE 7**Lymphoblastoid cell line SOD1 enzymatic activity¹**

	A4V FALS	Monosomy ² 21	Normal 2 x 21	Down's ² 3 x 21
SOD1 Activity	0.026	0.021	0.052	0.091
Relative SOD1 Protein ³	1.2	1.0	1.8	3.2
Activity/ relative SOD1 protein	0.021	0.021	0.029	0.028

¹SOD units/mg total protein²karyotype analysis demonstrated that these lines are monosomic and trisomic for all of chromosome 21 (D. Patterson, personal communication)³as assayed by Western blot

TABLE 8**Brain SOD1 activity¹**

	A4V FALS	Control
SOD1 Activity		
mean	223	392
n	3	12
std. dev.	33.5	100.3
Age		
mean	41.3	71.3
std. dev.	2.1	14.3
Sex Ratio (m/f)	2/1	7/5
Post-Mortem Interval		
mean	9.0	12.7
std. dev.	8.9	6.3

¹SOD₅₂₅ units/mg total protein

The mean SOD1 activity for the A4V FALS patients and the controls differ significantly ($p = 0.015$).

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1. A method of making a diagnostic for the detection of an increased likelihood of developing a cell death disease in a patient, said diagnostic comprising a means for analyzing the DNA of said patient to determine
5 whether said DNA contains a mutation in a SOD coding sequence, such a mutation being an indication that said patient has an increased likelihood of developing a cell death disease.

2. The method of claim 1, wherein said disease
10 is a neurodegenerative disease.

3. A method of claim 2, wherein said neurodegenerative disease is amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, Alzheimer's disease, Hallervorden-Spatz disease, or
15 olivopontocerebellar atrophy.

4. The method of claim 1, wherein said analysis is carried out by nucleotide sequencing, SSCP analysis, or RFLP analysis.

5. The method of claim 1, wherein said analysis
20 comprises amplifying a SOD-encoding gene of said patient using a SOD-specific primer, and then analyzing said amplified gene.

6. The method of claim 5, wherein said amplifying is carried out by PCR reaction, or reverse
25 transcriptase PCR.

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7. The method of claim 5, wherein said primer is derived from SOD1 nucleic acids, SOD2 nucleic acids, or SOD3 nucleic acids.

8. A kit for the diagnosis of a cell death
5, disease in a patient, said kit comprising one or more SOD gene-specific PCR primers.

9. The kit of claim 8, wherein a said PCR primer includes at least one of a SOD1-specific nucleic acid sequence, a SOD2-specific nucleic acid sequence, or a
10 SOD3-specific nucleic acid sequence.

10. A method of making a diagnostic for the detection of a disease involving a mutant SOD-encoding gene in a fetus, said diagnostic comprising a means for analyzing the DNA of said fetus to determine whether said
15 DNA contains a mutation in a SOD coding sequence.

11. A method of claim 10, wherein said SOD sequence from said fetus is compared to a SOD coding sequence of a relative of said fetus.

12. A method of making a diagnostic for the
20 detection of an increased likelihood of developing a cell death disease in a patient, said diagnostic comprising a means for analyzing the DNA of said patient to determine whether said DNA contains a mutation in an glutathione peroxidase coding sequence, such a mutation being an
25 indication that said patient has an increased likelihood of developing a cell death disease.

13. A method of making a diagnostic for the detection of an increased likelihood of developing a cell death disease in a patient, said diagnostic comprising a

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means for analyzing the DNA of said patient to determine whether said DNA contains a mutation in an catalase coding sequence, such a mutation being an indication that said patient has an increased likelihood of developing a cell death disease.

14. A method of making a diagnostic for the detection of an increased likelihood of developing a cell death disease in a patient, said diagnostic comprising a means for analyzing the DNA of said patient to determine whether said DNA contains a mutation in an nitric oxide synthase coding sequence, such a mutation being an indication that said patient has an increased likelihood of developing a cell death disease.

15. An antibody reactive with a FALS polypeptide but substantially unreactive with a wild-type SOD polypeptide.

16. The antibody of claim 15, wherein said antibody is a monoclonal antibody.

17. The antibody of claim 15, wherein said antibody is a polyclonal antibody obtained by subtractive techniques.

18. The antibody of claim 15, said antibody being capable of inactivating of said polypeptide.

19. A method of making a diagnostic for the detection of ALS in a patient, said diagnostic comprising a means for contacting a blood sample of said patient with the antibody of claim 15.

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20. A kit for the diagnosis of cell death disease, said kit comprising one or more SOD polypeptide specific antibodies.

21. Use of an antibody of claim 15 in the preparation of a medicament for treatment of a disease in a patient, said disease involving a mutant SOD-encoding gene.

22. Use of a SOD polypeptide in the preparation of a medicament for treatment of a patient with ALS.

23. Use of an antibody reactive with a mutant SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a mutant SOD-encoding gene.

24. The use of claim 23, wherein said antibody is obtained by subtractive techniques.

25. Use of an antioxidant in the preparation of a medicament for treatment of a disease in a patient, said disease involving a mutant SOD-encoding gene.

26. The use of claim 25, wherein said antioxidant is at least one of vitamin C, vitamin E, lycopene, bilirubin, urate, glutathione, dimercaprol, lutein, ubiquinol-10, dithiothretol, a mercaptan, a sulfa compound, methionine, cysteine or N-acetyl cysteine.

27. The use of claim 25, wherein said antioxidant is vitamin E.

28. The use of claim 25, wherein said antioxidant is vitamin C.

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29. The use of claim 25, wherein said antioxidant is a lazaroid.

30. The use of claim 25, wherein said antioxidant is Beta-carotene.

5 31. The use of claim 25, wherein said antioxidant is urate.

32. The use of claim 25, wherein said antioxidant is BHA.

10 33. The use of claim 25, wherein said antioxidant is BHT.

34. The use of claim 26, wherein said mercaptan is mercaptoethanol.

35. The use of claim 26, wherein said sulfa compound is a sulfonamide.

15 36. Use of a SOD polypeptide in the preparation of a medicament for treatment of a patient with a disease, said disease involving a mutant SOD-encoding gene.

20 37. The use of claim 36, wherein said SOD polypeptide is fused to a compound which specifically binds affected cells of said patient.

38. The use of claim 36, wherein said SOD polypeptide is at least one of Cu/ZnSOD, mSOD, or ecSOD.

25 39. Use of a chelating agent in the preparation of a medicament for treatment of a disease in a patient,

- 62 -

said disease involving a mutant SOD-encoding gene, said medicament effective to reduce the symptoms of said disease in said patient.

40. The use of claim 39, wherein said chelating agent is at least one of desferoxamine, EDTA, EGTA, DETC, BCDA, penicillamine, tetracycline, a metallothionein protein, or a apo-metal binding protein.

41. The use of claim 40, wherein said metallothionein protein is yeast copper metallothionein.

42. The use of claim 40, wherein said apo-metal binding protein is at least one of apo-superoxide dismutase, hemoglobin, myoglobin, or plastocyanin.

43. Use of at least one DNA oligonucleotide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a mutant SOD-encoding gene, said DNA oligonucleotide being substantially homologous to either the wild-type SOD gene or a mutant SOD gene.

44. The use of claim 43, wherein said oligonucleotide sequence encodes at least one of the Cu/ZnSOD polypeptide, mSOD polypeptide, or ecSOD polypeptide.

45. Use of the antisense DNA homolog of a gene encoding a SOD polypeptide in the preparation of a medicament for treating a patient with a disease, said disease involving a mutant SOD-encoding gene, and said preparation comprising manufacture of

a) a diagnostic for identifying a mutant SOD polypeptide-encoding gene in the DNA of said patient, and

- 63 -

b) a therapeutic amount of the said RNA.

46. The use of claim 45, wherein said SOD polypeptide is a wild-type SOD.

47. The use of claim 45, wherein said SOD
5 polypeptide is the polypeptide encoded by the mutant SOD-encoding gene.

48. Use of a transgene encoding the wild-type homolog of said mutant SOD polypeptide in the preparation of a medicament for treating a patient with a disease,
10 said disease involving a mutant SOD-encoding gene, and said preparation comprising manufacture of

a) a diagnostic for identifying a mutant SOD polypeptide-encoding gene in the DNA of said patient, and
b) a therapeutic amount of said transgene.

15 49. Use of a transgene encoding the anti-sense homolog of wild-type SOD RNA in the preparation of a medicament for treating a patient with a disease, said disease involving a mutant SOD-encoding gene, and said preparation comprising manufacture of

20 a) a diagnostic for identifying a mutant SOD polypeptide-encoding gene in the DNA of said patient, and
b) a therapeutic amount of said transgene.

50. The use of claim 48, wherein said transgene encodes a fusion polypeptide comprising, in addition to
25 said SOD polypeptide, a peptide which specifically binds affected cells of said patient.

51. Use of an inhibitor of wild-type SOD in the preparation of a medicament for treatment of a disease in

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a patient, said disease caused at least in part by excess SOD activity.

52. The use of claim 51, wherein said inhibitor is at least one of diethyldithiol carbonate or bathocurprone disulfonic acid.

53. Use of a mutant SOD polypeptide with increased SOD enzymatic activity compared to wild-type SOD in the preparation of a medicament for treatment of a patient with a disease involving a deleterious mutation in a SOD-encoding gene.

54. Use of a nucleotide sequence encoding a non-wild-type therapeutic SOD polypeptide different from and capable of inhibiting said deleterious SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in a SOD-encoding gene.

55. The use of claim 54, wherein said non-wild type therapeutic SOD is at least one of a fragment of SOD, an analog of SOD, or a non-peptide mimetic of SOD.

56. A use of a compound which participates in a biochemical pathway involving a SOD polypeptide in the preparation of a medicament for the treatment of a patient with a disease involving a mutant SOD-encoding gene.

57. The use of claim 56, wherein said compound is at least one of glutathione peroxidase, catalase, or nitric oxide synthase.

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58. Use of a FALS polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a neoplasm.

59. Use of a transgene encoding an FALS polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a neoplasm.

60. Use of a compound which is an inhibitor of a compound which participates in a biochemical pathway involving a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a mutant SOD-encoding gene.

61. Use of a compound capable of inhibiting an enzyme system associated with free radical production in the preparation of a medicament for treatment of a disease in a patient, said disease involving a mutant SOD-encoding gene.

62. Use of a peroxide-reducing polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a mutant SOD-encoding gene.

63. Use of an antioxidant in the preparation of a medicament for treatment of a disease in a patient with ALS.

64. Use of a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient with ALS.

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65. Use of a chelating agent in the preparation of a medicament for treatment of a disease in a patient having ALS.

5 66. Use of transgene comprising a nucleotide sequence encoding a SOD polypeptide in the preparation of a medicament for treatment of a patient having ALS.

67. Use of a mutant SOD polypeptide with increased SOD enzymatic activity compared to wild-type SOD in the preparation of a medicament for treatment of a
10 patient having ALS.

68. Use of a compound which participates in a biochemical pathway in the preparation of a medicament for treatment of a patient having ALS, said pathway involving a SOD polypeptide.

15 69. Use of a therapeutic amount of the anti-sense RNA homolog of a gene encoding a wild-type SOD polypeptide in the preparation of a medicament for treatment a patient having ALS.

20 70. Use of an inhibitor of SOD in the preparation of a medicament for treatment of a patient having ALS.

71. Use of a compound capable of inhibiting an enzyme system associated with free radical production in the preparation of a medicament for treatment of a patient having ALS.

25 72. Use of a peroxide-reducing polypeptide in the preparation of a medicament for treatment of a patient having ALS.

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73. Use of an antioxidant in the preparation of a medicament for treatment of a patient having a disease, said disease involving a deleterious mutation in the glutathione peroxidase-encoding gene.

5 74. Use of a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the glutathione peroxidase-encoding gene.

10 75. Use of a chelating agent in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the glutathione peroxidase-encoding gene.

15 76. The use of a transgene comprising a nucleotide sequence encoding a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the glutathione peroxidase-encoding gene.

20 77. Use of an antibody reactive with a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the glutathione peroxidase-encoding gene.

25 78. Use of a mutant SOD polypeptide with increased SOD enzymatic activity compared to wild-type SOD in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the glutathione peroxidase-encoding gene.

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79. Use of a compound which participates in a biochemical pathway involving a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation
5 in the glutathione peroxidase-encoding gene.

80. Use of a therapeutic amount of the anti-sense RNA homolog of a gene encoding a wild-type SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease
10 involving a deleterious mutation in the glutathione peroxidase-encoding gene.

81. The use of an inhibitor of SOD in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation
15 in the glutathione peroxidase-encoding gene.

82. Use of a compound capable of inhibiting an enzyme system associated with free radical production in the preparation of a medicament for treatment of a disease in a patient having a disease, said disease
20 involving a deleterious mutation in the glutathione peroxidase-encoding gene, said medicament effective to reduce the symptoms of said disease in said patient.

83. Use of a peroxide reducing polypeptide in the preparation of a medicament for treatment of a
25 disease in a patient, said disease involving a deleterious mutation in the glutathione peroxidase-encoding gene.

84. Use of an antioxidant in the preparation of a medicament for treatment of a disease in a patient, said

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disease involving a deleterious mutation in the catalase-encoding gene.

85. Use of a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient,
5 said disease involving a deleterious mutation in the catalase-encoding gene.

86. Use of a chelating agent in the preparation of a medicament for treatment of a disease in a patient,
10 said disease involving a deleterious mutation in the catalase-encoding gene.

87. The use of a transgene comprising a nucleotide sequence encoding a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation
15 in the catalase-encoding gene.

88. Use of an antibody reactive with a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the catalase-encoding
20 gene.

89. Use of a mutant SOD polypeptide with increased SOD enzymatic activity compared to wild-type SOD in the preparation of a medicament for treatment of a disease in a patient, said disease involving a
25 deleterious mutation in the catalase-encoding gene.

90. Use of a compound which participates in a biochemical pathway involving a SOD polypeptide in the preparation of a medicament for treatment of a disease in

- 70 -

a patient, said disease involving a deleterious mutation in the catalase-encoding gene.

91. Use of a therapeutic amount of the anti-sense RNA homolog of a gene encoding a wild-type SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the catalase-encoding gene.

92. Use of an inhibitor of SOD in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the catalase-encoding gene.

93. Use of a compound capable of inhibiting an enzyme system associated with free radical production in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the catalase-encoding gene.

94. Use of a peroxide-reducing polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the catalase-encoding gene.

95. Use of an antioxidant in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the nitric oxide synthase-encoding gene.

96. Use of a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the nitric oxide synthase-encoding gene.

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97. Use of a chelating agent in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the nitric oxide synthase-encoding gene.

5 98. Use of a transgene comprising a nucleotide sequence encoding a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the nitric oxide synthase-encoding gene.

10 99. Use of an antibody reactive with a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the nitric oxide synthase-encoding gene.

15 100. Use of a mutant SOD polypeptide with increased SOD enzymatic activity compared to wild-type SOD in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation in the nitric oxide synthase-
20 encoding gene.

 101. Use of a compound which participates in a biochemical pathway involving a SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease involving a deleterious mutation
25 in the nitric oxide synthase-encoding gene.

 102. Use of a therapeutic amount of the anti-sense RNA homolog of a gene encoding a wild-type SOD polypeptide in the preparation of a medicament for treatment of a disease in a patient, said disease

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involving a deleterious mutation in the nitric oxide synthase-encoding gene.

103. Use of an inhibitor of SOD in the preparation of a medicament for treatment of a disease in
5 a patient, said disease involving a deleterious mutation in the nitric oxide synthase-encoding gene.

104. Use of a compound capable of inhibiting an enzyme system associated with free radical production in the preparation of a medicament for treatment of a
10 disease in a patient, said disease involving a deleterious mutation in the nitric oxide synthase-encoding gene.

105. Use of a peroxide-reducing polypeptide in the preparation of a medicament for treatment of a
15 disease in a patient, said disease involving a deleterious mutation in the nitric oxide synthase-encoding gene.

106. The use of any one of claims 61, 93, 104, 71, or 82 wherein said drug is at least one of deprenyl
20 or a lazaroid.

107. The use of any one of claims 94, 105, 72, or 83 wherein said peroxide-reducing polypeptide is at least one of selenium dependent glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase,
25 cytochrome c peroxidase, ascorbate peroxidase, NAD(P)H peroxidase, guaiacol peroxidase, ceruloplasmin, albumin, transferrin, ferritin, a reductase or a thiol-protein.

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108. The use of claim 111, wherein said reductase is any one of glutathione reductase, monodehydroascorbate reductase or dehydroascorbate reductase.

109. A transgenic non-human animal whose somatic and germ cells contain a transgene coding for a disease-causing mutant SOD polypeptide.

110. An animal of claim 109, wherein said animal is a mouse.

111. An animal of claim 109, wherein said animal is a worm.

112. A bacterial cell containing purified nucleic acid derived from a FALS gene.

113. A yeast cell containing purified nucleic acid derived from a FALS gene.

114. Purified DNA encoding a purified FALS polypeptide.

115. Purified RNA encoding a purified FALS polypeptide.

116. A purified FALS polypeptide.

117. The use of any one of claims 25, 36, 39, 43, 44, 45, 48, 10, 21, 23, 51, 53, 54, 56, 61, 62, wherein said disease is amyotrophic lateral sclerosis.

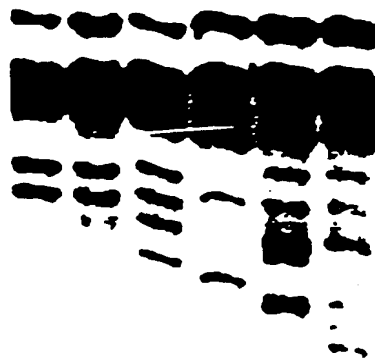
118. The use of any one of claims 3, 25, 36, 39, 43, 44, 45, 48, 10, 21, 23, 51, 53, 54, 56, 61, 62, wherein said amyotrophic lateral sclerosis is familial.

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FIGURE 1 A

Exon 2

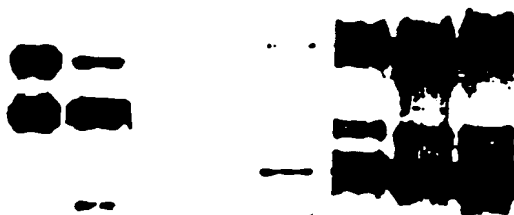
N N 11 18 33 36



Exon 4

N 3

N 57 130 118



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EXON 2

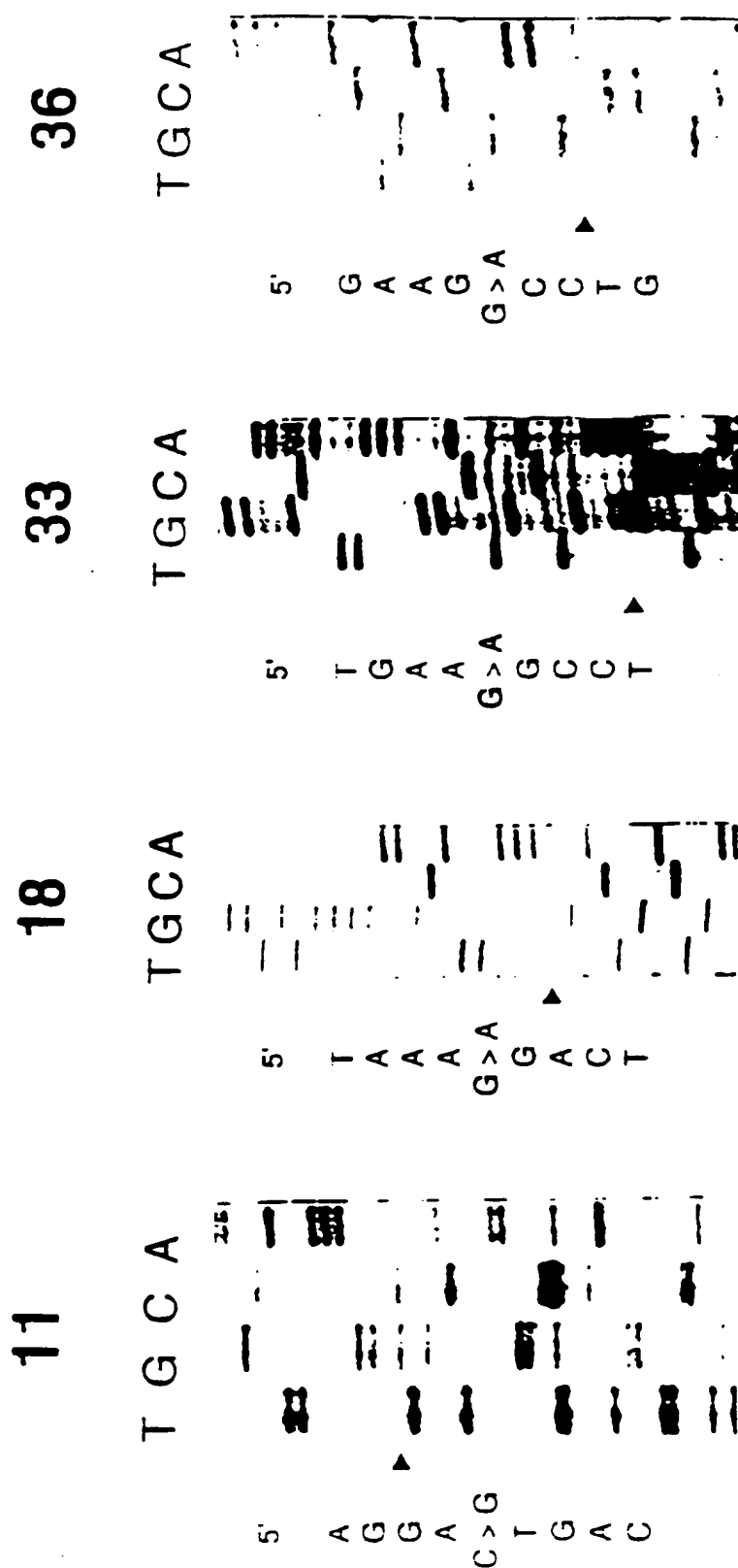


FIGURE 1 B

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EXON 4

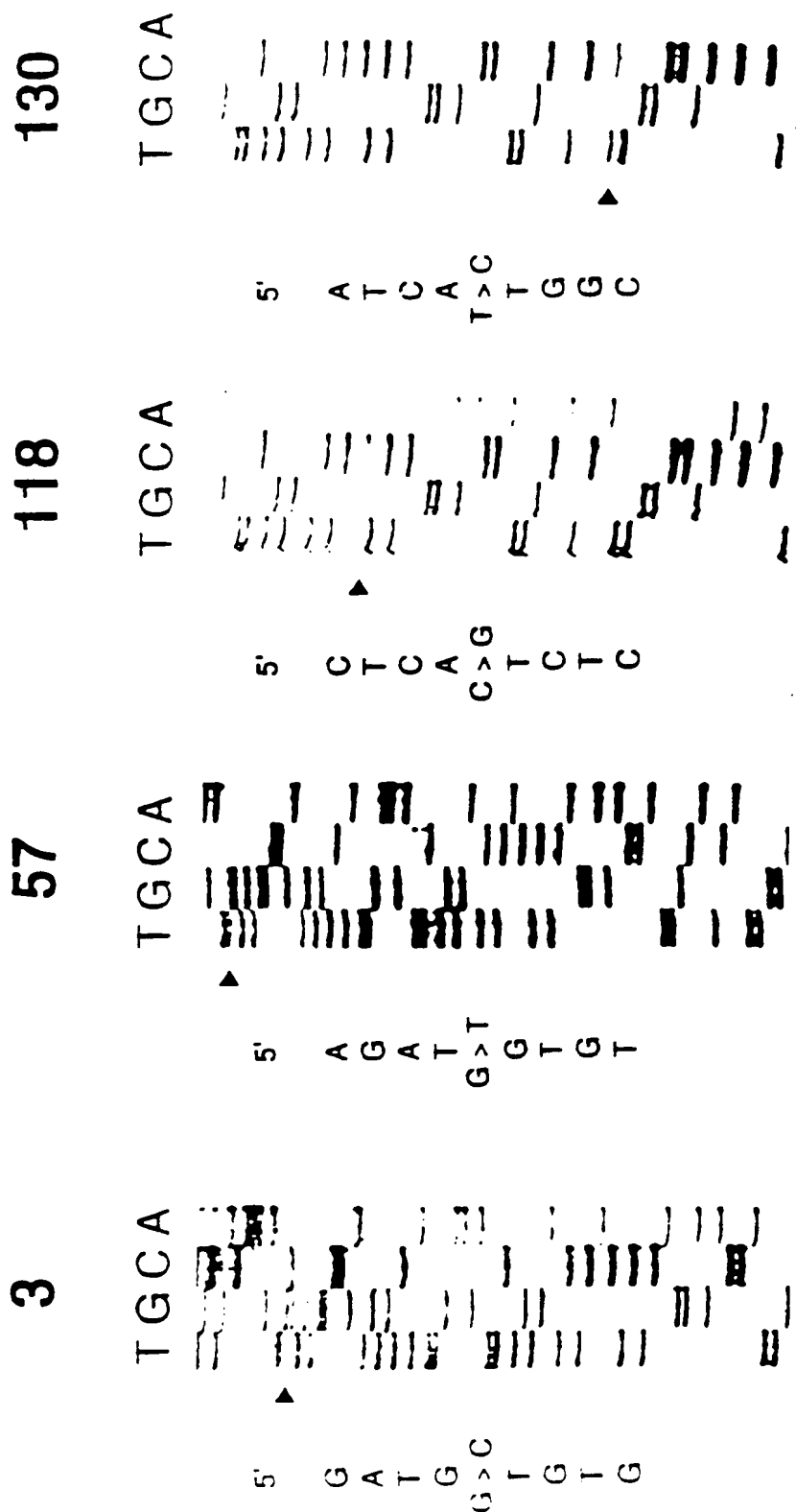


FIGURE 1 B (CONT'D)

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Figure 2. Sequence of Human SOD-1

-552 GAATTCTGCC AACCAATTA GAACTCTAT ACTAAGGACT AAGAAAATTG (SEQ ID NO: 1)
-502 CAGGGGAAGA AAAGGTAAGT CCCGGGATTG AGGTCTAGCG ACTTTCTATA
-452 CCCTCAGAAA ACTAAAAAAC AAGACAAAAA AATGAAAAC TACAAAAGCAT
-402 CCATCTTGGG GCGTCCCAAT TGCTGAGTAA CAAATGAGAC GCTGTGGCCA
-352 AACTCACGTC ATAAC TAATG ACATTTCTAG ACAAAGTGAC TTCAGATTTT
-302 CAAAGCGTAC CTTGTTTACA TCATTTTGCC AATTTCGCGT ACTGCAACCG
-252 GCGGGCCACG CCCCCCTGAA AAGAAGGTTG TTTTCTCCAC ATTTCCGGGT
-202 TCTGGACGTT TCCCCGCTGC GGGGCGGGGG GAGTCTCCCG CGCACGCGGC
-152 CCCTTGGCCC CGCCCCCAGT CATTCGCGGC CACTCGCGAC CCGAGGCTGC
-102 CGCAGGGGGC GGGCTGAGCG CGTGCGAGGC GATTGGTTTG GGGCCAGAGT
-52 GGGCGAGGCG CGGAGGTCTG GCCTATAAAG TAGTCGCGGA GACGGGGTGC
-2 TG
1 GTTTGCGTCC TAGTCTCCTG CAGCGTCTGG GGTTCCTCTT GCAGTCCTCG
51 GAACGAGGAC CTCGGCTTGG CCTAGCGAGT T ATG GCG ACG AAG GCG
met ala thr lys ala
97 GTG TGC GTG CTG AAG GGC GAC GGC CCA GTG CAG GGC ATC ATC
6 val cys val leu lys gly asp gly pro val gin gly ile ile
139 AAT TTC GAG CAG AAG GCAAGGG CTGGGACGGG AGGCTTGTGG
20 asn phe glu gin lys intron 1
TTGCGAGGCG GCTCCCCACG CGCTCGTCCC CCGCGGACCG TTTGCATGGA
CGGGTCGCCC GCCAGGG.....
CCTAGAGCAG GTTAAGCAGC TTGCTGGAGG TTCACTGGCT AGAAAGTGGT
CAGCCTGGGA TTTGGACACA GATTTTTCOA CTCCCAAGTC TGGCTGCTTT
TACTTCACT GTGAGGGGTA AAGGTAAATC AGCTGTTTTG TTTGTTTACA
AAGTCTCTCC AACTTTGAC TTTTCTTAAA G

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FIGURE 2 (CONT'D)

154 GAA AGT AAT GGA CCA GTG AAG GTG TGG GGA AGC ATT AAA GGA (SEQ ID NO:
 36 glu ser asn gly pro val lys val trp gly ser ile lys gly (continued)

196 CTC ACT GAA GGC CTC CAT GGA TTC CAT GTT CAT GAG TTT GGA
 40 leu thr glu gly leu his gly phe his val his glu phe gly

238 GAT AAT ACA GCA 3 GTCCGGTGTT.....
 54 asp asn thr ala intron 2

GTGTTTCTTT TTAGAATGTA TTGCGGAAC TTAATTCATA ATTTAGCTTT

TTTTTCTTCT TCTTATAAAT AG

251 GC TGT ACC AGT GCA GGT CCT CAC TTT AAT CCT CTA TCC AGA
 58 gly cys thr ser ala gly pro his phe asn pro leu ser arg

292 AAA CAC GGT GGC CCA AAG CAT GAA GAG AG GTAACAGAT
 72 lys his gly gly pro lys asp glu glu arg intron 3

GCTTAACCTCT TGTAAATAATg gccGATCATG GTTCTGGAGT TCATATGGTA

TACTACTTGT AAATATGTGC TAAGATAATT CCTGTGTTCC CCCACCTTTG

CTTTTGAAC TGCCTGACTCA TCTAAACCT GCTCCCAAAT GCTGGAATGC

TTTTACTTCC TGGGCTTAAA GGAATTGACA AATGGGCACT TAAAACGATT

TGGTTTTGTA GCATTGATT GAATATAGAA CTAATACAAG TGCCAAAGGG

GAACATAATAC AGGAAATGTT CATGAACAGT ACTGTCAACC ACTAGCAAAA

TCAATCATCA TT.....

GTACTTCTGA AATCAGGTGC AGCCCCATCT TTCTTCCCAG AGCATTAGTG

TGTAGACGTG AAGCCTTGTT TGAAGAGCTG TATTTAGAAT GCCTAGCTAC

TTGTTTGCAA ATTTGTGTCC TACTCAGTCA AGTTTAAATT TAGCTCATGA

ACTACCTTGA TGTTTAGTGg CATCAGCCCT AATCCATCTG ATGCTTTTTC

ATTATTAG

321 3 CAT GTT GGA GAC TTG GGC AAT GTG ACT GCT GAC AAA GAT
 32 his val gly asp leu gly asn val thr ala asp lys asp

361 GGT GTG GCC CAT GTG TCT ATT GAA GAT TCT GTG ATC TCA CTC
 35 gly val ala asp val ser ile glu asp ser val ile ser leu

403 TCA GGA GAC CAT TGC ATC ATT GGC GGC ACA CTG GTG
 109 ser gly asp his cys ile ile gly arg thr leu val

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FIGURE 2 (CONT'D)

GTAAG TTTTCATAAA AGGATATGCA TAAAACTTCT TCTAACATAC (SEQ ID NO: 1
 intron 4 (continued)

AGTCATGTAT CTTTTCACCTT TGATTGTTAG TCGCGGTTTC TAAGATCCAG
 ATAAACTGT.....
 GAAAAAGCTT TGAGTAGTAG TTTCTACTTT TAAACTACTA AATATTAGTA
 TATCTCTCTA CTAGGATTAA TGTTATTTTTT CTAATATTAT GAGGTTCTTA
 AACATCTTTT GGGTATTGTT GGGAGGAGGT AGTGATTACT TGACAGCCCA
 AAGTTATCTT CTTAAAATTT TTTACAG

444 CTC CAT GAA AAA GCA GAT GAC TTG GGC AAA GGT GGA AAT GAA
 101 val his glu lys ala asp asp leu gly lys gly gly asn glu

486 GAA AGT ACA AAG ACA GGA AAC GCT GGA AGT CGT TTG GCT TGT 135
 glu ser thr lys thr gly asn ala gly ser arg leu ala cys

528 GGT GTA ATT GGG ATC GCC CAA TAA ACATTCCCT TGGATGTAGT
 149 gly val ile gly ile ala gln

CTGAGGCCCC TTAACATC TGTTATCCTG CTAGCTGTAG AAATGTATCC
 TGATAAACAT TAAACACTGT AATCTTAAAA GTGTAATTGT GTGACTTTTT
 CAGAGTTGCT TTAAAGTACC TGTAGTGAGA AACTGATTTA TGATCACTTG
 GAAGATTTGT ATAGTTTTAT AAAACTCAGT TAAAATGTCT GTTTC AATGA
 CCTGTATTTT GCCAGACTTA AATCACAGAT GGGTATTAAA CTTGTCAGAA
 TTTCTTTGTC ATTCAAGCCT GTGAATAAAA ACCCTGTATG GCACCTATTA
 TGAGGCTATT AAAAGAATCC AAATTCAAAC TAAATTAGCT CTGATACTTA
 TTTATATAAA CTGCTTCAGT GGAACAGATT TAGTAATACT AACAGTGATA
 GCATTTTATT TTGAAAGTGT TTTGAGACCA TCAAAATGCA TACTTTAAAA
 CAGCAGGTCT TTTAGCTAAA ACTAACACAA CTCTGCTTAG ACAAATAGGC
 TGTCCCTTGA AGCTT

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Figure 3. Sequence of Human SOD-2

1 CCGCCCGCCG CCAGGAGCCG CACTCTGGC TGTGGTGGCT TCGGCAGCCG (SEQ ID NO: 2)

51 CTTTACGAGA TCGGCGGCAT CAGCGGTACG ACCAGCACTA GCAGC ATG
met

99 TTG AGC CCG GCA CTG TGC GGC ACC AGC AGG CAG CTG GCT CCG
leu ser arg ala val cys gly thr ser arg gln leu ala pro

141 GCT TTG GGG TAT CTG GGC TCC AGG CAG AAG CAC AGC CTC CCC
ala leu gly tyr leu gly ser arg gln lys his ser leu pro

183 GAC CTG CCG TAC GAC TAC GGC GCC CTG GAA CCT CAC ATC AAC
asp leu pro tyr asp tyr gly ala leu glu pro his ile asn

225 GCG CAG ATC ATG CAG CTG CAC CAC AGC AAG CAC CAC CCG GCC
ala gln ile met gln leu his his ser lys his his ala ala

267 TAC CTG AAC AAC CTG AAC CTC ACC GAG CAG AAG TAC CAG GAG
tyr val asn asn leu asn val thr glu glu lys tyr gln glu

309 GCG TTG GCA AAG GGA GAT GTT ACA GCC CAG ACA GCT CTT CAG
ala leu ala lys gly asp val thr ala gln thr ala leu gln

351 CCT GCA CTG AAG TTC AAT GGT GGT GGT CAT ATC AAT CAT AGC
pro ala leu lys phe asn gly gly gly his ile asn his ser

393 ATT TTC TGG ACA AAC CTC AGC CCT AAC GGT GGT GGA GAA CCC
ile phe trp thr asn leu ser pro asn gly gly gly glu pro

435 AAA GGG GAG TTG CTG GAA GCC ATC AAA CGT GAC TTT GGT TCC
lys gly glu leu leu glu ala ile lys arg asp phe gly ser

477 TTT GAC AAG TTT AAG GAG AAG CTG ACC GCT GCA TCT GTT GGT
phe asp lys phe lys glu lys leu thr ala ala ser val gly

519 GTC CAA GGC TCA GGT TGG GGT TGG CTT GGT TTC AAT AAG GAA
val gln gly ser gly trp gly trp leu gly phe asn lys glu

561 CCG GGA CAC TTA CAA ATT GCT GCT TGT CCA AAT CAG GAT CCA
arg gly his leu gln ile ala ala cys pro asn gln asp pro

603 CTG CAA GGA ACA ACA GGC CTT ATT CCA CTG CTG GGG ATT GAT
leu gln gly thr thr gly leu ile pro leu leu gly ile asp

645 GTG TGG GAG CAC GCT TAC TAC CTT CAG TAT AAA AAT GTC AGG
val trp glu his ala tyr tyr leu gln tyr lys asn val arg

687 GCT GAT TAT CTA AAA GCT ATT TGG AAT GTA ATC AAC TGG GAG
pro asp tyr leu lys ala ile trp asn val ile asn trp glu

729 AAT GTA ACT GAA AGA TAC ATG GCT TGC AAA AAG TAA ACCACG
asn val thr glu arg tyr met ala cys lys lys

771 ATCGTTATGC TGAGTATGTT AAGCTCTTTA TGACTCTTTT TGTAGTGGTA

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FIGURE 3 (CONT'D)

821 TAGAGTACTG CAGAATACAG TAAGCTGCTG TATTGTAGCA TTTCTTGATG (SEQ ID NO: 2)
871 TTGCTTAGTG ACTTATTTCA TAAACAACCT AATGTTCTGA ATAATTTCTT (continued)
921 ACTAAACATT TTGTTATTGG SCAGTGAAT GAAAATAGTA AATGCTTTGT
971 GTGATTG

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Figure 4. Sequence of Human SOD-3

1 CTGGGTCCAG CTCTCTTTTC AGGAGAGAAA CCTCTCTTTGG AGGAGCTGGA (SEQ ID NO: 3
(continued)

51 AAGGTGCCCC ACTCCAGCC ATG CTG GCG CTA CTG TGT TCC TCC
met leu ala leu leu cys ser cys

94 CTG CTC CTG GCA GCG GGT GCG TCG GAC GCG TCG ACC GGC GAG
leu leu leu ala ala gly ala ser asp ala trp thr gly glu

136 GAC TCG GCG GAG CCC AAC TCT GAC TCG GCG GAG TCG ATC CGA
asp ser ala glu pro asn ser asp ser ala glu trp ile arg

178 GAC ATG TAC GCC AAG GTC ACG GAG ATC TCG CAG GAG GTC ATG
asp met tyr ala lys val thr glu ile trp gln glu val met

220 CAG CCG CCG GAC GAC GAC GCG ACC CTC CAC GCG GCG TCG CAG
gln arg arg asp asp asp gly thr leu his ala ala cys gln

262 CTG CAG CCG TCG GCG ACC CTC GAC GCG GCG CAG CCC CCG GTG
val gln pro ser ala thr leu asp ala ala gln pro arg val

304 ACC GGC GTC GTC CTC TTC CCG CAG CTT GCG CCC CCG GCG AAG
thr gly val val leu phe arg gln leu ala pro arg ala lys

346 CTC GAC GCC TTC TTC GCC CTG GAG GCG TTC CCG ACC GAG CCG
leu asp ala phe phe ala leu glu gly phe pro thr glu pro

388 AAC AGC TCC AGC CCG GCC ATC CAC GTG CAC CAG TTC GGG GAC
asn ser ser ser arg ala ile his val his gln phe gly asp

430 CTG AGC CAG GCG TCG GAG TCC ACC GCG CCC CAC TAC AAC CCG
leu ser gln gly cys glu ser thr gly pro his tyr asn pro

472 CTG GCC GTG CCG CAC CCG CAG CAC CCG GCG GAC TTC GGC AAC
leu ala val pro his pro gln his pro gly asp phe gly asn

514 TTC GCG GTC CCG GAC GCG AGC CTC TCG AGG TAC CCG GCC GCG
phe ala val arg asp gly ser leu trp arg tyr arg ala gly

556 CTG GCC GCC TCG CTC GCG GCG CCG CAC TCC ATC GTG GCG CCG
leu ala ala ser leu ala gly pro his ser ile val gly arg

598 GCC GTG GTC GTC CAC GCT GCG GAG GAC GAC CTC GCG CCG GCG
ala val val val his ala gly glu asp asp leu gly arg gly

640 GCG AAC CAG GCC AGC GTG GAG AAC GCG AAC GCG GCG CCG CCG
gly asn gln ala ser val glu asn gly asn ala gly arg arg

682 CTG GCC TCG TCG GTG GTG GCG GTG TCG GCG CCC GCG CTC TCG
leu ala cys cys val val gly val cys gly pro gly leu trp

724 CAG CCG CAG GCG CCG GAG CAC TCA GAG CCG AAG AAG CCG CCG
glu arg gln ala arg glu his ser glu arg lys lys arg arg

766 TCG CAG AGC CAG TCG AAG GCG GCG TCA GCGGCGCC CCCACCCCGC
arg glu ser glu cys lys ala ala

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FIGURE 4 (CONT'D)

811 GCGCGCCAGG GACCCCGGAG GCGCCCTCTT GCCTTTGAGC TTCTCCTCTG (SEQ ID NO: 3)
(continued)

861 CTCCTTCCAG CACCTTCCAG TCTGAGGTCT CACCTTCCGC TCTGCTGAAG

911 TCTCCCGCCA GCGCTCTCCA CCGAGAGGTC TCGCTATAGC GAGACCCAGC

961 ATCCTTCCAT CCTGAGGAGC GCGCCAACCC TCGGAGCCCC CCACTCAGTA

1011 GGTCTGAAGG CCTCCATTTC TACCGAAACA CCGCCCTCAC GCTGACAGCC

1061 TCCTAGGCTC CCTGAGGTAC CTTTCCACCC AGACCCCTCT TCCCCACCCC

1111 ATAAGCCCTG AGACTCCCGC CTTTGACCTG ACGATCTTCC CCCTTCCCGC

1161 CTTCAGGTTT CTCCTAGGCG CTCAGAGGCC GCTCTGGGGG GTTGCCTCGA

1211 CTCCTCCCGC CCGTCCCGAC CCAACCACCC TCGCCCGGCA AGCCAGCCCC

1261 TCGAACGGAA CCGAGGCCAA CTCCTCCCGC TCTTCAGCTG TTTCGCATCC

1311 ACCGCCACCC CACTGAGAGC TCGTCTTTTC GGGGAATGTT TGGCAACCTT

1361 TGTGTTACAG ATTAAAAATT CAGCAATTC

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Figure 5. PCR Primers for Human SOD-1

Exon 1

5' ATA AAG TAG TCG CGG AGA CGG 3'

5' GCC TTC TGC TCG AAA TTG ATG 3'

Exon 2

5' ACT CTC TCC AAC TTT GCA CTT 3'

5' CCC ACC TGC TGT ATT ATC TCC 3'

Exon 3

5' GAA TGT ATT TGG GAA CTT TAA TTC 3'

5' TAG ATG AGT CAG CAA GTT CAA AAG 3'

Exon 4

5' CAT ATA GGC ATG TTG GAG ACT 3'

5' GAA AGA TAC ATG ACT GTA CTG 3'

Exon 5

5' GTA TTG TTG GGA GGA GGT AGT GAT 3'

5' GCA GGA TAA CAG ATG AGT TAA GGG 3'

Figure 6. PCR Primers for RT-PCR of
Human SOD-2 and SOD-3

SOD-2

5' GCA ACA TCA AGA AAT GCT AC 3'

5' GGC ACT CGT GGC TGT GGT GGC TTC 3'

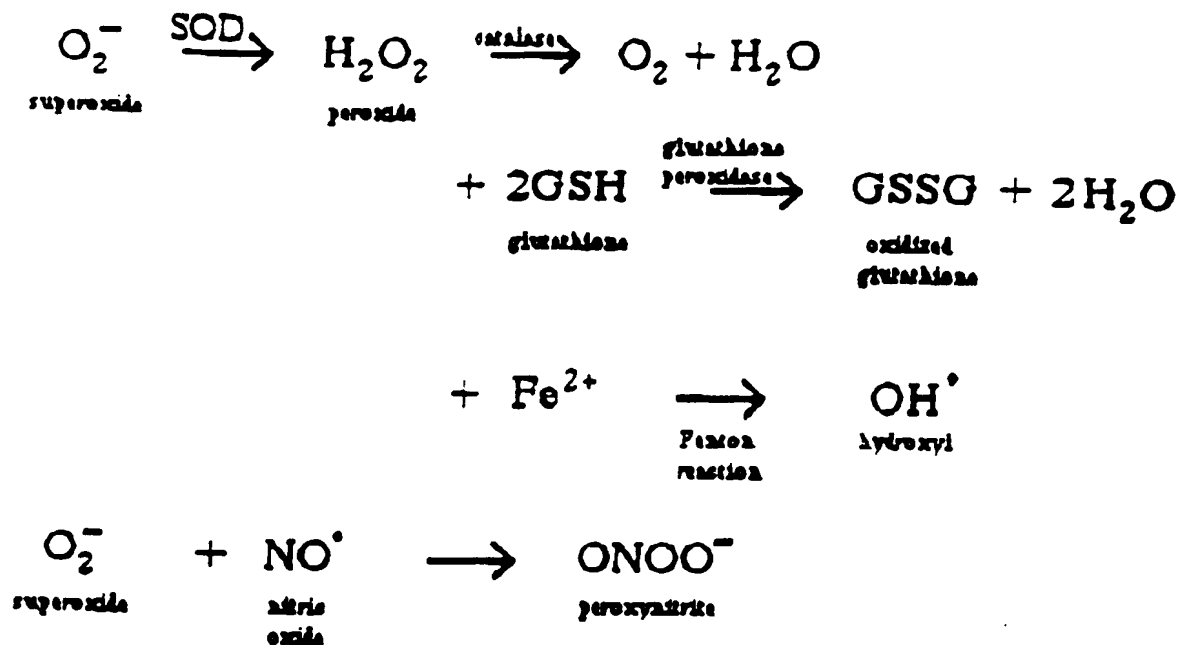
SOD-3

5' CAC AAA GGT AGC CAA ACA TTC 3'

5' GTG CAG CTC TCT TTT CAG GAG 3'

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Figure 7. Oxygen Free Radicals and Their Reactions



Exon 1

N 127



FIGURE 8 A

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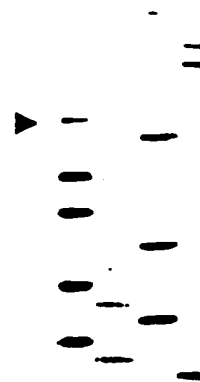
FIGURE 8 B

EXON 1

127

TGCA

5'
A
A
G
G
C > T
C
G
T
G



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FIGURE 8 C

Amino Acid Sequences in SOD1 Exon 1

EXON 1

FALS		V	
human	(M)A T - K A V C V L K G D G P V Q G I I N F E Q K		
cow	(M)A T - K A V C V L K G D G P V Q G T I H F E A K		
pig	A T - K A V C V L K G D G P V Q G T I Y F E L K		
mouse	(M)A M - K A V C V L K G D G P V Q G T I H F E Q K		
<i>Xenopus</i>	V - K A V C V L A G S G D V K G V V R F E Q Q		
swordfish	V L - K A V C V L R G A G E T T G T V Y F F E Q E		
<i>Drosophila</i>	(M)V V - K A V C V I N G D A K - - G T V F F E Q E		
tomato	(M)V - K A V A V L N S S E G V S G T Y L F T Q V		
spinach	A T K K A V A V L L G T S N V E G V V T L T Q E		
maize	(M)V - K A V A V L A G T - D V K G T I F F S Q E		
<i>Neurospora</i>	(M)V - K A V A V V R G D S N V K G T V I F F E Q E		
<i>S. cerevisiae</i>	V - Q A V A V L K G D A G V S G V V K F F E Q A		

FIGURE 9



B.

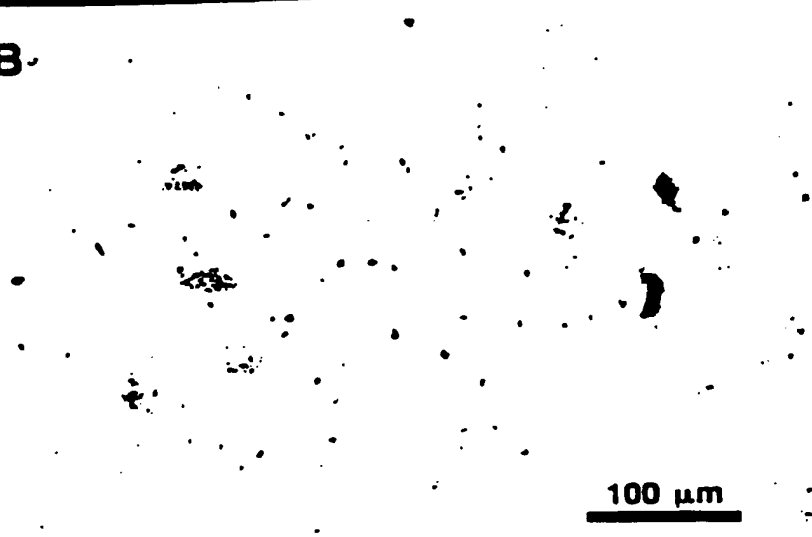


FIGURE 10

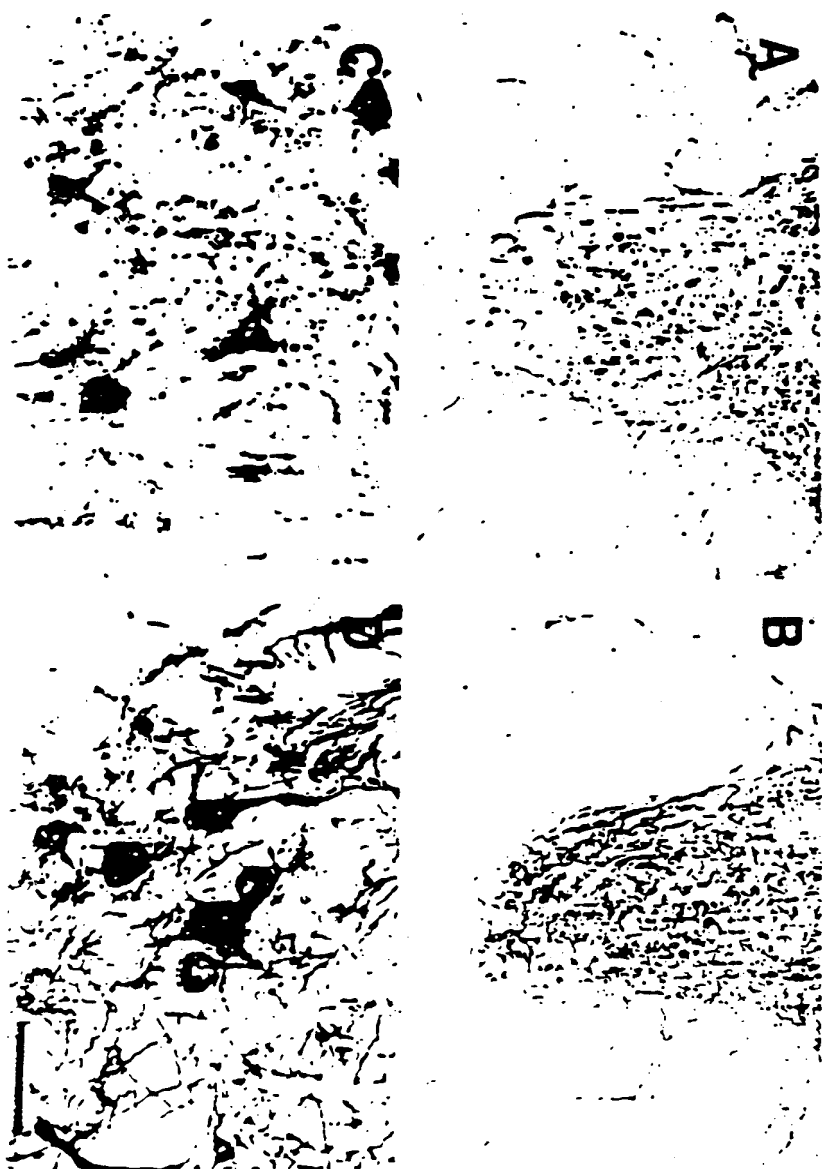


FIGURE 11

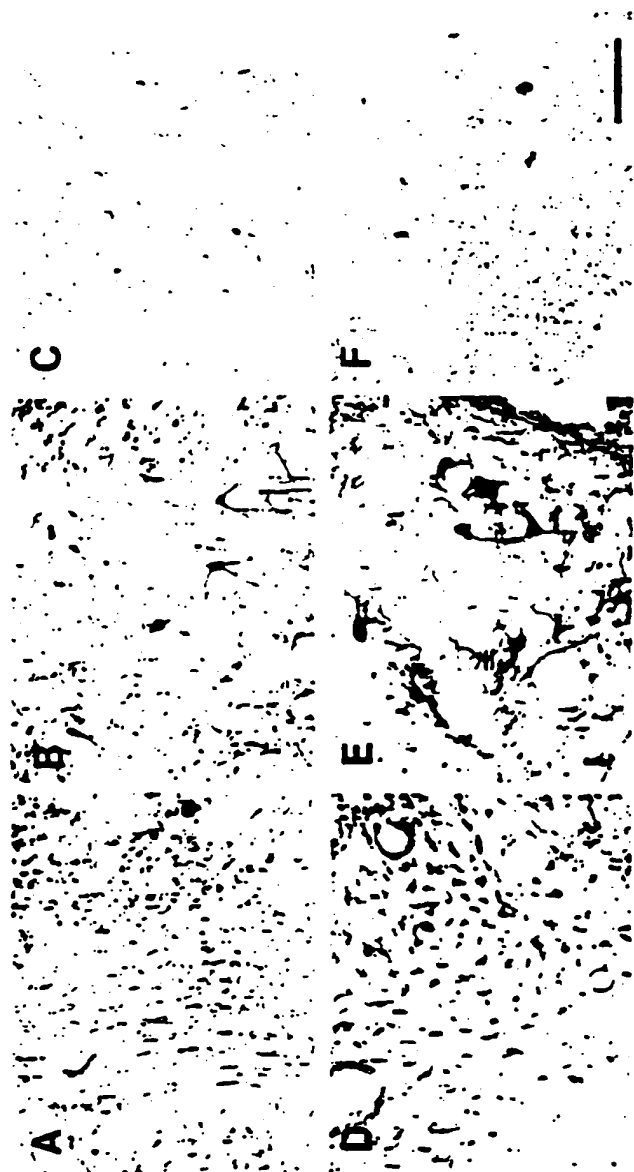


FIGURE 12

EXON 4

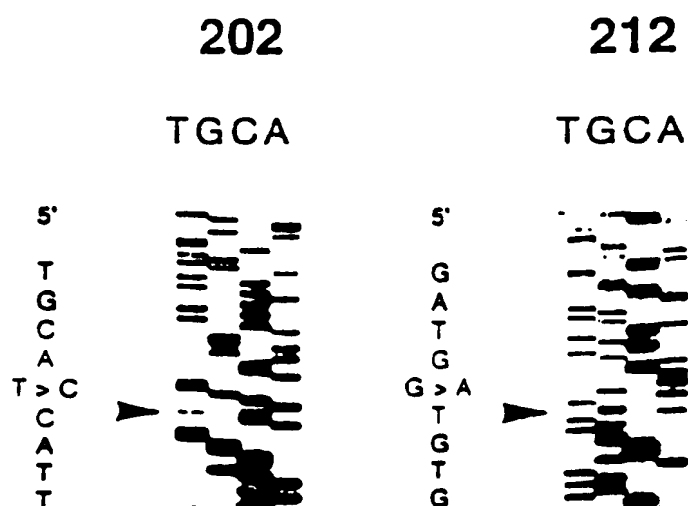
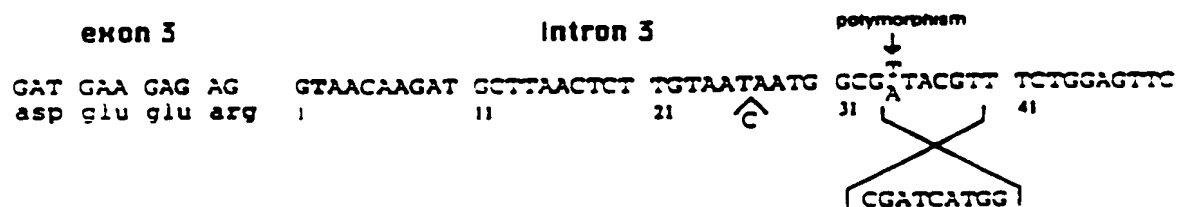


FIGURE 13

Polymorphism in Intron 3 of SOD1



A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12Q 1/68; C12P 19/34; C07H 21/04

US CL : 435/6, 91.2; 536/24.33,

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/24.33,

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Biosis

search terms: SOD, fetus, neurodegenerative disease, diagnosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ----- Y,P	The Lancet, Volume 342, issued 23 October 1993, Brock et al., "Cu/Zn superoxide dismutase (SOD1) mutations and sporadic amyotrophic lateral sclerosis", pages 1050-1051, see entire document.	1-9 ----- 10-11
X,P ----- Y,P	Nature Genetics, Volume 5, issued December 1993, Ogasawara et al., "Mild ALS in Japan associated with novel SOD mutation", pages 323-324, see entire document.	1-9 ----- 10-11
X,P ----- Y,P	Nature, Volume 362, issued 04 March 1993, Rosen et al., "Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis", pages 59-62, see entire document.	1-9 ----- 10-11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

20 MAY 1994

Date of mailing of the international search report

JUN 01 1994

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/02089

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ----- Y,P	Science, Volume 261, issued 20 August 1993, Deng et al., "Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide sidmutase", pages 1047-1051, see entire document.	1-9 ----- 10-11

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1-11, drawn to diagnostic methods involving mutant SOD genes.
- II. Claim 12, drawn to diagnostic methods involving mutant glutathione peroxidase genes.
- III. Claim 13, drawn to diagnostic methods involving mutant catalase genes.
- IV. Claim 14, drawn to diagnostic methods involving mutant nitric oxide synthase genes.
- V. Claims 15-20, drawn to antibodies against mutant SOD polypeptides.
- VI. Claims 21-24, 36-38, 53, 64-65, 67, 74, 77-78, 85, 88-89, 96, 99-100, and 117-118, drawn to methods of treating diseases using SOD polypeptides or antibodies.
- VII. Claims 25-35, drawn to methods of treating diseases using antioxidants.
- VIII. Claims 39-42, 75, 86, and 97, drawn to methods of treating diseases using chelating agents.
- IX. Claims 43-50, 54-55, 66, 69, 76, 80, 87, 91, 98, and 102, drawn to methods of treating diseases using nucleic acids.
- X. Claims 51-52, 70, 81, 92, and 103, drawn to methods of treating diseases using inhibitors.
- XI. Claims 56-57, 60-63, 68, 71-73, 79, 82-84, 90, 93-95, 101, and 104-108, drawn to methods of treating diseases using compounds which participate in a biochemical pathway involving SOD.
- XII. Claims 58-59, drawn to methods of treating a neoplasm using a FALS polypeptide or nucleic acid encoding said polypeptide.
- XIII. Claims 109-111, drawn to transgenic non-human animals.
- XIV. Claims 112-116, drawn to purified FALS nucleic acids, bacterial or yeast cells containing said nucleic acids, and a purified FALS polypeptide.

and it considered that the International Application did not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The claims of these groups are directed to different inventions which are not linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general concept. The claims in the different groups do not have in common the same or corresponding "special technical features". PCT Rules 13.1-13.3 provide for grouping of claims drawn to a product, process of manufacture of said product, and use of said product, for example, but do not provide for grouping of multiple distinct products and methods. Diagnostic methods are distinct from methods of treatment; further, the above treatments involve different molecules requiring completely different considerations, such as the use of antibodies, nucleic acids, or metabolic inhibitors. Thus, PCT Rules 13.1-13.3 do not provide for the grouping together of the above distinct inventions.